Induction of Resistance to Viral Infections in the Domestic Cat by Stimulation of the Innate Immune System

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Abbreviations

ABCD	Advisory Board on Cat Diseases
AUC	Area Under the Curve
CID	Cat Infectious Dose
CpG	Cytosine Phosphate Guanosine
CPV	Canine Parvovirus
CrFK	Crandell Feline Kidney Cells
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
ds	double-stranded
FCV	Feline Calicivirus
FCoV	Feline Coronavirus
fcwf-4	Felis Catus Whole Fetus Cells
FEA	Feline Embryonic Fibroblasts
FeLV	Feline Leukaemia Virus
FIP	Feline Infectious Peritonitis
FIV	Feline Immunodeficiency Virus
FHV	Feline Herpes Virus
FPV	Feline Parvovirus
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GUSB	Beta Glucoronidase
HBSS	Hank's Balanced Salt Solution
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
IFN	Interferon
IFNαR	Interferon Alpha Receptor
IL	Interleukin
IRF	Interferon Regulatory Factor
IRM	Immune Response Modifier
ISG	Interferon Stimulated Gene
MDA5	Melanoma Differentiation Associated Gene 5
MHC	Major Histocompatibility Complex
MMR	Measles, Mumps, Rubella
Mx	Myxovirus Resistance Protein
NFκB	Nuclear Factor Kappa B
NK	Natural Killer
OAS	Oligoadenylate Synthetase
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid Dendritic Cell
PKR	RNA-dependent Protein Kinase
PRR	Pathogen Recognition Receptor
rfeIFNα	Recombinant Feline Interferon Alpha
RLR	(RIG-I)-like Receptor
RIG	Retinoic Acid Inducible Gene
RNA	Ribonucleic Acid
SARS	Severe Acute Respiratory Sydrom
ss	single-stranded
TCID	Tissue Culture Infectious Dose
Th	T Helper Cell
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
VSV	Vesicular Stomatitis Virus
WHO	World Health Organization
YWHAZ	Tryptophan 5-monooxygenase Activation Protein Zeta Polypeptide

1. Summary

Broadening the understanding of mechanisms linked to innate immunity is of primordial importance in a time of continuous emergence of rapidly spreading viral diseases. The domestic cat represents an ideal model for the study of host-virus interactions, as it is an outbred species naturally susceptible to many viruses sharing biological properties with those affecting humans. Additionally, due to their acquisition of infallible transmission strategies, rapid propagation of feline viruses within a group is particularly difficult to inhibit, reflecting the challenges linked to the prevention of pandemics. The present work was designed to gain insights on the innate immune responses of the domestic cat to viruses, and to determine whether early antiviral mechanisms can be manipulated to enhance resistance to viral infection in this species.

In a first phase, real-time polymerase chain reaction (PCR) systems were developed, enabling to measure the expression of feline genes considered to be hallmarks of innate responses to viral infection, including various interferon (IFN)α and IFNω subtypes, IFNβ, intracellular antiviral Myxovirus resistance (Mx) factor, natural killer (NK) cell stimulator IL-15 and effectors perforin and granzyme B, as well as Toll-like receptors (TLRs) 3, 7, 8 and 9. These tools could then be employed to evaluate innate immune parameters in both *in vitro* and *in vivo* models of infection, conferring valuable information not only regarding strength, breadth and kinetics of antiviral defences in feline cells, but also possible biological properties of important viruses affecting the cat.

In a further step, the newly developed PCR assays were utilized to assess the immunomodulatory potential of various immune response modifiers (IRMs) in feline cells *in vitro*. The IRMs mimicking natural viral components were selected, namely Poly IC and Resiquimod (R-848), artificial models of viral dsRNA and ssRNA, as well as dSLIM™ and ODN 2216, synthetic oligonucleotides containing several unmethylated CpG motifs. Although all analysed IRMs positively modulated the innate immune state of treated peripheral mononuclear cells (PBMCs), ODN 2216 induced by far the most potent response: this molecule not only altered the gene expression profile of feline PBMCs in an antiviral orientation, but also significantly enhanced the proliferation of these immune cells and increased the presence on their surface of co-stimulatory molecules necessary for the diffusion of immunological defence signals. Moreover, when incubated in vitro with target cells of epithelial and fibroblastic origin,

the supernatants of ODN 2216-stimulated PBMCs not only induced high production of intracellular antiviral proteins in these cells but also inhibited the replication of five feline viruses, namely the feline calici- (FCV), herpes- (FHV), parvo- (FPV), corona- (FCoV) and leukemia (FeLV) viruses.

Altogether, this study procures a better understanding of innate antiviral mechanisms in an outbred species and highlights the promising potential of CpG-containing molecules such as ODN 2216 to protect domestic cats against a broad range of virus infections. Further *in vitro* and *in vivo* investigations will determine the feasibility of stimulation of the innate immune system by such molecules to prevent viral propagation in humans, cats and other species.

Zusammenfassung

Infolge der immer häufigeren Ausbrüche noch unbekannter, sogenannter „emerging" viralen Krankheiten, wird es heutzutage immer wichtiger, das Verständnis der Mechanismen des angeborenen Immunsystems zu verbessern. Für solchen Studien ist die Hauskatze ein ideales Modell, da sie ausgezüchtet und empfänglich für verschiedene Viren ist, die nahe verwandt sind mit denjenigen die auch Menschen betreffen. Aehnlich wie im Falle einer Pandemie ist die schnelle Ausbreitung viraler Infektionen in einer Gruppe von Katzen zudem schwierig zu verhindern, da feline Viren während der Evolution effiziente Uebertragungsstrategien entwickelt haben. Die Ziele der vorliegenden Arbeit umfassten das Erwerben neuer Erkenntnisse über das angeborene Immunsystem der Katze und die Prüfung der Möglichkeit zur Induktion einer allgemeinen Resistenz gegen virale Infektionen in dieser Spezies durch die Stimulierung antiviraler Immunmechanismen.

In einem ersten Schritt wurden real-time Polymerase Ketten Reaktion (PCR) Systeme entwickelt, mit denen die Expression von felinen Genen gemessen werden können, die im Ablauf einer angeborenen Immunantwort gegen Virusinfektionen eine Rolle spielen. Dazu gehören verschiedene Interferon (IFN) α and IFNω Subtypen, IFNβ, der intrazelluläre antivirale Myxovirus Resistenz (Mx) Faktor, das (Natural Killer) NK Zell stimulierende Zytokin IL-15 sowie NK Effektorproteine Perforin and Granzyme B, und die Toll-like Rezeptoren (TLR) 3, 7, 8 und 9. In einer zweiten Phase wurden diese

neuen Assays eingesetzt, um die angeborenen Immunparameter in Infektionsmodellen *in vitro* und *in vivo* zu evaluieren. Die Resultate lieferten wertvolle Angaben über die Stärke, Breite und Kinetik von antiviralen Abwehrmechanismen in felinen Zellen. Die weitere Anwendung der entwickelten PCR Systemen erlaubte das immunmodulierende Potential verschiedener sogenannten „immune response modifiers“ (IRMs) *in vitro* in Katzenzellen zu evaluieren. Es wurden IRMs ausgewählt, welche natürlichen viralen Komponenten ähnlich sind, nämlich Poly IC und Resiquimod (R-848), synthetische Varianten der viralen doppelsträngigen (ds) RNA and einzelsträngigen (ss) RNA, sowie dSLIM™ und ODN 2216, synthetische Oligonukleotide, die unmethylierten Cytosine-Phosphat-Guanosine (CpG) Motive beinhalten. Obwohl alle analysierten IRMs den Immunstatus von stimulierten peripheren mononukleären Zellen (PBMCs) positiv beeinflussten, erwies sich ODN 2216 als der stärkste IRM: dieses Molekül induzierte nicht nur die Expression von antiviralen Effektorgenen in felinen PBMCs, sondern stimulierte auch die Proliferation dieser Immunzellen und erhöhte die Präsenz auf deren Oberfläche von costimulatorischen Molekülen, welche für die Verbreitung von antiviralen Signalen äusserst wichtig sind. Zudem induzierten Ueberstände, welche *in vitro* durch ODN 2216-stimulierte PBMCS produziert worden waren, die Produktion von intrazellulären antiviralen Mx Proteinen in Zielzellen aus epithelialem und fibroblastischem Ursprung, womit die Replikation von fünf felinen Viren markant gehemmt wurde. Bei den Viren handelt es sich um die felinen Calici- (FCV), Herpes- (FHV), Parvo- (FPV), Corona- (FCoV) und Leukaemie. (FeLV) Viren, die alle zu verschiedenen Familien gehören.

Insgesamt vermittelt diese Studie ein besseres Verständnis der angeborenen antiviralen Mechanismen in einer ausgezüchteten Spezies und zeigt das vielversprechende Potenzial von CpG-enthaltenden Molekülen die Hauskatze gegen ein breites Spektrum von Virusinfektionen zu schützen. Mit weiteren *in vitro* und *in vivo* Untersuchungen sollte die Möglichkeit geprüft werden, die Uebertragung von Virusinfektionen in Menschen, Katzen, und anderen Spezies zu hemmen.

2. Introduction

Close to 7 billion people and more than 600 million domestic cats (*felis catus*) inhabit our planet. These two populations have evolved alongside one another for thousands of years rendering today's Tom, Sylvester and Garfield the results of a long respectful relationship between very independent entities. Despite the cat's relatively little contribution to human survival in comparison to other species originally domesticated for their milk, meat, wool or labour capabilities, recent genetic and archaeological discoveries indicate that cats and humans have been associated for at least 9,500 years [1].

How did humans and cats come to share common paths? Each side probably had individual interests in cohabitation. In 2009, Driscoll and colleagues traced the common ancestry of the cat back to the wildcat of the Middle East, *felis sylvestris lybica* [2]. Most likely due to restricted food basis, this ancestor of today's domestic cat was obliged to lead a solitary way of life and defend its hunting territories from other cats. With the emergence of early human settlements however, a new environment with abundance of food allowed cats to live in groups: both the house mouse (*Mus musculus domesticus*) that quickly populated the settlements and the trash dumped on the outskirts of towns most likely prompted the cats to live among humans. In turn, the inhabitants probably did not mind the company of the cats, considering not only that they were discrete and caused no harm, but also that they eliminated mice and snakes form the villages. Some experts additionally speculate that wild cats naturally possess features such as large eyes, small faces and high round foreheads that are known to elicit nurturing from humans and might facilitate the development of a relationship with people [1]. Today, cats occupy all continents, and have gained the status of the most popular pet on the planet [1].

One downside linked to the domestication of cats remains the dispersion of specific microorganisms. In order to survive in their host population, feline viruses in particular have adapted to the solitary way of life of cats over hundreds of thousands of years. Thus, they possess both the capability to induce latent and/or asymptomatic infections and very efficient transmission strategies enabling them to infect susceptible individuals

upon the rare contact between animals [3]. In view of the opportunistic behaviour of feline viruses, it is not difficult to imagine that the advent of domestication gave a free way to these pathogens, which readily spread, as a consequence, to every individual living in a group. As it is currently accepted that the biology of virus-host interactions is a continuous co-evolutionary process involving both host immune system and viral escape mechanisms [4], the survival of the domestic cat in the course of evolution suggests the presence or selection of strong antiviral defence mechanisms in this species. The immunological factors responsible for this evolutionary retaliation against fatal viruses however, remain poorly understood to date.

Viruses remain a threat to human and animal populations worldwide, killing millions of individuals each year. Unfortunately, the elaboration of specific medical care against viral infections has proven to be particularly tricky, as viruses possess highly developed adaptation faculties. Simple mutational alterations result in resistant strains that considerably limit the potential of existing antiviral drugs, rendering prophylactic measures especially important in the combat against viruses. In view of developing new approaches to prevent viral propagation, many research groups worldwide currently focus on characterising the early interactions of viruses with the immune system. It just so happens that felids are naturally affected by members of many different viral families, that share biological properties with pathogens held responsible for pandemics not only in humans, but also in other animal populations (for further detail see section 2.3.2) [5]. Thus, a better understanding of the immunological antiviral defence mechanisms of the domestic cat may not only support the development of more efficient prophylactic measures against feline viruses, but also provide new insights on host and virus specific factors that play a significant role in pathogenesis of important human and animal diseases. The cat may indeed play a much more important role in its relationship with humans than running after mice and providing a cuddly face…

Along these lines, the present work sheds light on fundamental questions linked to immune responses of the domestic cat and further defines this species as an alternative model in studies related to host-virus interactions. Two manuscripts included in chapter 3 of this document summarize the main developments and findings of this project. In order to support their understanding by the reader and to provide a more detailed

perspective in the relevance of this study, current facts concerning host-virus relationships of human and feline populations as well as conventional and alternative preventive methods are delineated as background information hereafter (section 2.1). The objectives of the present study are then clearly formulated in section 2.2. Finally, although results are extensively discussed in the respective manuscripts included in this document, the overall outcome of this study is thoroughly examined in the final discussion (chapter 5), which also opens perspectives for future experiments.

2.1 Background and state of research in the field

2.1.1 You, me, viruses…

In 1901, Walter Reed was the first to imply that viruses could induce disease in humans, when he discerned that the devastating yellow fever plague that hit America in the late 19th century was caused by a viral pathogen. Since then, viruses have been held responsible for millions of deaths worldwide, even starting hundreds of years before their discovery. Although rapid advances in technology during the past century have enabled to identify, treat, prevent, and sometimes even eradicate viruses, the list of theses dangerous pathogens keeps growing posing major challenges to human health. According to the World Health Organization (WHO), up to one third of all deaths worldwide each year are linked to viral diseases.

In the last century, vaccination has been developed and used with fantastic success as preventive measure against many viral infections. Today, children are immunized at a very young age with the MMR vaccine, which combines protection to viruses causing measles, mumps, and rubella. Since introduction of the earliest versions of MMR in the 1970s, over 500 million doses have been used in more than 60 countries worldwide. Vaccination against poliovirus enabled to eradicate poliomyelitis, a devastating disease affecting the nervous system, from the Western hemisphere in the second half of the 20th century. Hepatitis A and B vaccines furthermore confer protection against severe virally-induced liver disease. The herpes zoster vaccine helps reduce the risk of shingles, the painful rash caused by the reactivation of a member of the herpes virus family causing chickenpox, and the more recently developed human papillomavirus (HPV) vaccine helps protect against cervical cancer and genital warts induced by this

virus. Although the development of an effective and safe antiviral vaccine is a notoriously long and costly process governed by stringent regulatory controls typically lasting 8 to 12 years and costing between $300 and $800 million, vaccination most often elicits strong, specific immune responses that confer long-term protection.

The currently most dangerous viral pathogens tend to be the "emerging" and "re-emerging viruses", which are defined by the WHO as "newly recognised, newly evolved or occurred previously but have shown an increase in incidence or expansion of geographical, vector or host range" and for which consequently no vaccination is available. "Emerging" viruses most often enter the human population sporadically from animal reservoirs and include those pathogens responsible for the severe acute respiratory syndrome (SARS), avian influenza, and the Nipah encephalitis. These viruses are mostly unrecognized and of unknown origin when they appear in a population, or have undergone uncharacterised mutations giving an open path to their rapid dissemination before effective prevention strategies can be developed. "Re-emerging" viruses such as West Nile, Chikungunya and Dengue viruses, the Japanese Encephalitis virus and the Human Immunodeficiency virus (HIV) are most often vector borne (transmitted by flies and mosquitoes) or, as in the case of HIV, sexually transmitted. These pathogens are known and sometimes endemic, but may emerge again to cause more outbreaks and in places not previously known for outbreaks to occur. Most emerging and re-emerging viruses cause severe human disease and possess dangerous pandemic potential. In those cases when other animal populations like poultry or pigs may also be affected, additional issues arise including economic losses related to the culling of animals and the unavailability of food due to real or suspected contamination. These risks have the potential to severely disrupt global food supply chains and further harm human health and welfare.

The influential factors contributing to the current appearance and dissemination of new viruses are linked to societal-based decisions and demographic changes that are generally considered to be a direct consequence of today's human economic development [6]. Thus, specific environmental conditions coupled with the socio-economic influences of today have defined "emerging disease hotspots" in lower latitudes, including tropical Africa, Latin America, and Asia [7]. Countries in this

region bear a rapidly growing population density and yet inadequate hygiene conditions and education, which together greatly facilitate the spread of communicable and sexually transmitted diseases. A high birth rate moreover increases the amount of immunologically naïve individuals who perpetuate epidemics. The typical warm, humid climate in affected countries promotes vector-borne diseases and the development of rice agriculture creates both ecological niches for insects and breeding environments for water birds that are natural reservoirs of various emerging and re-emerging diseases. The potential for pathogens to cross species barriers is exacerbated by extensive livestock production, persistence of livestock trading markets and deforestation, which altogether promote interactions between human beings, livestock and wildlife. Moreover, due to increasing global transportation of goods and people, viral infections that would have been confined to a limited geographical area can easily spread worldwide. Finally, these changes in population dynamics and economic activities lead to a great loss in biodiversity, which has recently been linked to enhanced transmission of disease [8].

Outbreaks of emerging and re-emerging viral diseases are generally accompanied by devastating public health and economic consequences. In the event of a pandemic, not only many human and animal lives are at stake, but industries in life science, food and health care sectors are highly exposed to considerable deficits. Other industries suffer economic losses due to reduction in workforces as well as decrease in public gatherings, travel, and tourism. Presently, the most feared global emerging disease scenario internationally is the influenza pandemic. In 1918, influenza virus was responsible for an estimated 50 million deaths worldwide, and a new influenza pandemic is expected when the virus mutates to a form that is readily transmitted among humans. Current epidemiological models of the WHO project that a pandemic could kill as many as 10 million people worldwide and losses to global economies could top $1 trillion. These dark prospects assuredly pressure the development of new preventive methods to impair the dissemination of emerging viruses. An effective prophylaxy would induce broad protection against a wide selection of viruses, as emerging viral microorganisms cannot be specifically targeted due to their unpredictable nature. The development of novel preventive strategies to emerging viral diseases remains a great challenge in medical research today.

2.1.2 Cats and viruses

The domestic cat is a natural host of several viruses that infect the feline population worldwide, along with wild felids and in some cases other wild carnivores (for references see individual virus sections below). Feline viruses have acquired over the course of evolution elaborate strategies to persist within their host population. In order to convey a basic idea about the relationship between the cat and its viruses, the six feline viruses commonly encountered in the field will be briefly portrayed in this section, with a focus not only on the disease they cause in the cat, but also on structural and epidemiological properties that render them particularly challenging to combat. Experiments conducted in the present project and described in the manuscripts of chapter 3 also include most of the feline viruses introduced here. For further information regarding these and other feline pathogens, fact sheets and guidelines have been issued by the European Advisory Board for Cat Diseases (ABCD) in 2009 and can be consulted online (www.abcd-vets.org).

2.1.2.1 Feline herpes virus

The feline herpes virus (FHV) is an enveloped virus with a double-stranded DNA (dsDNA) genome that belongs to the Varicellovirus genus and the subfamily Alphah*erpesvirinae*. Although the virulence can differ among strains, only one serotype is known and distributed in domestic cats and some wildcat populations worldwide [9]. The disease caused by feline herpes virus affects the upper respiratory tract and is often referred to as feline viral rhinotrachetitits or cat flu, which is characterized by conjunctivitis, profuse ocular and nasal discharges, and in some cases, severe keratitis and corneal ulceration. Co-infections with feline calicivirus and bacteria are not uncommon, causing a multi-agent respiratory syndrome with exacerbated symptoms [10]. Transmission occurs via oral, nasal or conjunctival routes and, as the virus is rapidly inactivated in the environment, requires direct contact with a shedding cat. Acute disease is accompanied by viral excretion for 1-3 weeks after which the clinical symptoms usually disappear. However, a latent/chronic course of disease is the typical outcome of an acute FHV infection due to neuronal latency of the virus, and affected cats become lifelong carriers. Intermittent reactivation of viral secretion can occur in carrier cats upon glucocorticoid treatment or during stressful events such as lactation or

moving into a new environment. Vaccines are available for domestic cats, however vaccination may not always prevent infection [11]. Furthermore, under some circumstances, vaccinated cats can still develop latent FHV-1 infections with consequent periodic reactivations which allow the virus to transmit [12]. Prevalence of disease in feline population varies in several studies from 1% in small healthy populations to 20% in shelters and multicat households with respiratory problems [13].

2.1.2.2 Feline calicivirus

The feline calicivirus (FCV) belongs to the Vesivirus Genus and the *Caliciviridae* family. This highly contagious pathogen is a non-enveloped virus consisting of a small single-stranded RNA (ssRNA) genome [14]. Although mainly restricted to cat populations, FCV presents a high genome plasticity, which allows the virus to respond rapidly to environmental selection pressures. Most isolates consequently present antigenic differences [15]. Acute infection typically leads to oral ulcers and upper respiratory disease with sneezing and nasal discharge. A virulent systemic form of FCV disease has also been reported [16]. As mentioned under 2.1.2.1, FCV is often associated with FHV and various bacterial strains in a common respiratory syndrome. Similarly to FHV, transmission occurs during direct contact with a shedding animal via the oral, nasal and conjunctival routes. FCV can persist for up to one month in the environment under dry conditions at room temperature, so that indirect transmission through contaminated objects can also occur [17]. The virus is shed not only during acute disease, but also for more than 30 days thereafter, and in some individuals for several years [18]. Vaccines against FCV are available, however several studies indicate the appearance of field strains that are resistant to vaccine-induced immune responses [19]. The prevalence of disease seems proportional to the number of cats in a household, ranging from around 10% in small groups up to 25-40% in colonies and shelters, with a high variability between individual colonies [20].

2.1.2.3 Feline panleukopenia virus

The feline panleukopenia virus (FPV) is a non-enveloped virus with a ssDNA genome, which belongs to the parvovirus genus and the *Parvoviridae* family. This virus is known as the prototype parvovirus of carnivores, and has been shown to infect other members

of the *felidae*, as well as racoons, minks and foxes [21]. Cats are additionally susceptible to canine parvovirus (CPV)-2 [22] so that superinfection and co-infection with multiple parvovirus strains may occur, potentially facilitating recombination and high genetic heterogeneity [23]. FPV is transmitted by the faecal-oral route, and distributed to rapidly dividing cells of the body including those of bone marrow, lymphoid tissues and intestinal crypts, as the virus requires cells in the S-phase of division for its replication. Consequently, immunosuppression, severe diarrhoea and dehydration are hallmark symptoms of infection. Mortality is extremely high, and can occur in up to 90% of infected kittens. Diseased carnivores shed virus at high titres and the virus displays resistance to both physical factors and chemical agents, leaving contaminated environments infectious for months and rendering indirect transmission the main mode of infection [24]. Vaccines against FPV are available on the market; however, this virus has re-emerged as a major cause of mortality in cats in shelters and rescue homes, most probably due to insufficient vaccination linked to budget reasons [25].

2.1.2.4 Feline coronavirus

The feline coronavirus (FCoV) is a large, spherical, enveloped ssRNA virus that belongs to the *Coronaviridae* family of the order Nidovirales. FCoV infection is ubiquitous in dometic cats (seroprevalence 20-100%) and wild felids may also be seropositive. Most cats are infected following contact with FCoV in faeces of asymptomatic cats, begin to shed virus within one week and continue to shed for weeks, months or even sometimes for life [26, 27]. FCoV can survive for 7 weeks in a dry environment and may be transmitted indirectly. While most FCoV positive cats are asymptomatic or display only mild enteritis, about 12% will eventually develop feline infectious peritonitis (FIP), a highly fatal pyogranulomatous vasculitis [28]. Pathologically, FIP has been classified into two forms: an effusive (wet) form characterised by polyserositis (eg, thoracic and abdominal effusion) and vasculitis, and a non-effusive (dry) form characterised by granulomatous lesions in various organs. Clinical symptoms linked to FIP are highly variable and often unspecific. Generally, the affected cat presents fever, anorexia and lethargy often accompanied by ascites in the wet form of FIP. Although the precise pathogenesis leading to the development of FIP

remains to be fully understood, it is believed that the continuous emergence of new phenotypes by mutation and recombination during replication of this large RNA virus enables it to spread efficiently in the host by circumventing natural specific immune responses [29]. Infectious viral load, viral genetics and the cat's individual immunity all play a role in determining whether FIP will develop [30]. A vaccine against FIP has been introduced, however with limited efficacy in the field, as most cats become infected many weeks before they can be vaccinated. Infection is particularly common in multi-cat environments and where conditions are crowded, whereas the prevalence is lower in single pets [31].

2.1.2.5 Feline leukemia virus

The feline leukemia virus (FeLV) belongs to the gammaretrovirus genus and the *Retroviridae* family. Infections with this virus occur worldwide, and in addition to domestic cats, FeLV has been detected in other small felids such as the wildcats (*felis silvestris*) and Iberian lynxes [32, 33]. Like other retroviruses, FeLV is an enveloped virus with a ssRNA genome that must be reverse transcribed and integrated as a "provirus" DNA form into the host's genome. Although they may or may not produce infectious virus, infected cells are consequently permanently infected and pass the virus on to daughter cells. Individual immunological factors that have yet to be identified determine whether the host cells of a particular cat will eliminate (20-30%), persistently produce (30-40%) or transiently replicate (30-40%) virus [34]. Clinical signs are mainly linked to persistent FeLV replication and include anaemia, immunosuppression and lymphoma; most affected cats die within 2-3 years. As FeLV does not survive for long periods outside the host, direct contact is usually required for effective infection of another cat. The virus is mainly transmitted through saliva, milk, faeces and nasal secretions during friendly contacts, but also through bites [35]. Vaccines against FeLV are available; however they do not prevent infection and thus cannot confer 100% efficacy of protection against disease. Indeed, minimal viral replication occurs in infected vaccinated cats, and provirus can be readily detected in these animals [36]. The prevalence of FeLV infections is highly influenced by the density of cat populations, and studies indicate a high geographical variability. In most developed countries the

prevalence in individually kept cats is usually less than 1%, while in multi-cat households lacking specific preventive measures it may exceed 20% [34].

2.1.2.6 Feline immunodeficiency virus

The feline immunodeficiency virus (FIV) belongs to the lentivirus genus and the *Retroviridae* family. FIV infection is endemic in domestic cat populations worldwide, and a variety of species-specific strains have been isolated from wild feline populations including the puma, lion, leopard and Pallas cat [37-40]. Like FeLV, FIV is an enveloped ssRNA virus that integrates in the host genome during its replication cycle. The viral reverse transcriptase that mediates transcription of the RNA genome into a DNA copy for its integration is error-prone and lacks a proofreading function, facilitating rapid mutation of the virus that displays great genetic diversity. Five genetically distinct FIV subtypes or clades have been defined that cluster in a geographic manner, indicating local adaptation of the virus [41-43]. Although FIV is closely related to the human immunodeficiency virus (HIV) and shares a similar structure, life cycle and pathogenesis, humans cannot be infected. Virus is shed in saliva and transmitted almost exclusively through bites. Older, free-roaming male cats are thus more prone to infection due to exhibition of territorial behaviour. Transmission from mother to kittens may occur, especially if the queen is undergoing early or late phases of infection. Once in contact with the blood of a host cat, FIV persistently infects CD4+ T lymphocytes, which play a central role in the development of both cellular and humoral immune responses to pathogens. Infected cats generally remain asymptomatic for several years, and most clinical signs are finally the consequence of immunodeficiency and secondary infection linked to the chronic loss of CD4+ T cells. A vaccine against FIV is commercially available in the USA, Australia and New Zealand. This whole inactivated virus preparation however does not protect against all different clades of FIV, and failed to induce protection against European FIV subtypes [44]. Several seroepidemiological surveys have revealed an overall seroprevalence worldwide of approximately 10% among both healthy and sick cats screened in North America, Asia, Europe and Oceania [45]. Cats living in environments with high population density belong to higher risk groups.

2.1.2.7 Feline viral strategies for persistence

As can be noted in the descriptive sections 2.1.2.1-2.1.2.6, various traits of feline viruses enable them to persist within the cat population and render their prevention very tricky. Most feline viruses apply the "hit and stay" strategy, in that they remain for a long time within the host after infection [46]. This is most likely the signature of viral adaptation to the individual way of life of the wild ancestor of today's domestic cat: feline viruses had to develop ways to both wait for contact between individuals and efficiently spread with high efficiency from one cat to another on this rare occasion (see introduction).

The astuteness of feline viruses relies on several mechanisms varying from induction of latency in the host and affection of a broad host range to persistence in the environment and rapid adaptation to external pressures. Latency of infection is common in FHV, FeLV and FIV, and infected cats are likely to renew viral shedding in stressful situations most often implying contact with other cats, such as mating, lactation and moving to new locations in which territories must be re-established. Additionally, animals infected with FCV, FCoV, and FeLV may remain chronic shedders for a lifetime, even in the absence of clinical signs of disease. This asymptomatic carrier state is of primordial importance in the epidemiology of feline viruses. FPV infection may be the exception to this rule, as dissemination of this virus relies more on a strategy known as "hit and run". Infected kittens are quickly killed during FPV infections, depleting the pool of susceptible individuals and presenting a higher risk of extinction for the virus. Thus, this pathogen has evolved compensatory methods to "stay around anyway" and wait for the next cat to come: infected animals shed extremely high amounts of virus, which is so resistant that it can persist in the environment for many months. Similarly, FCV and FCoV also exhibit significant resistance outside the host and persist in the environment as a threat for uninfected cats. Most feline viruses moreover exhibit a broad host range, infecting not only domestic cat populations, but also wild felids and, in the case of FPV, other carnivores. The possibility to affect wider populations increases chances both to encounter another susceptible individual and to disseminate over larger geographical areas. Another advantage of certain feline viruses resides in their genomic structure and replication characteristics. Indeed, RNA viruses generally possess high antigenic variability [47] and those affecting cats (FCV, FCoV, FeLV and

FIV) typically utilize this capability to escape immune responses and propagate more efficiently. Thus, the genetic diversity among viral strains across their geographic range, typically observed in FCV, FeLV and FIV, suggests local viral adaptations [48]. Finally, feline viruses support each other through narrow collaboration: FeLV and FIV both disarm the host from its systemic immune defences opening windows of opportunities for other members of the feline virus family, while FHV and FCV weaken local barriers of the upper respiratory tract and thus promote each other's proliferation. Altogether, the infectious strategies of feline viruses have enabled their survival in the feline population over evolution and additionally seem to drive them to adapt to current pressures induced by vaccines. No vaccination available to date fully protects domestic cats against these pathogens, and resistant strains of individual viruses are already emerging.

2.1.2.8 Where cats and viruses meet

Many studies have highlighted the role of the host population structure as a selection pressure for infectious pathogens [49, 50]. In concordance with this, field observations reveal a high variability in viral prevalence according to the cat social organisation and mating system: viruses thrive in large groups with narrow contacts [48]. Indeed, over the course of time, feline viruses seem to have conserved their opportunistic behaviour: efficient transmission strategies enable them to infect virtually every cat living in a group.

It is consequently not surprising that multi-cat households, breeding facilities, shelters and rescue homes are the preferred places for feline viruses to "hang out" [10, 51, 52]. Close proximity and high social contact rates among individuals from different backgrounds, continuous resident turnover and stressful environments are characteristic for most multi-cat households, shelters and rescue homes. In large colonies, communal rearing of kittens by females also enhances viral spread. As the viruses remain "hidden" within asymptomatic or healthy carriers for long periods of time, there is unfortunately often no apparent urgent need for prophylactic measures. Furthermore, since clinical symptoms of many feline viral diseases appear only long after the time point of infection, the relation to a stay at a breeding facility or a shelter is often overseen.

Unfortunately, it is also in shelters and rescue homes, where antiviral prevention is of primordial importance, that vaccines may not systematically be employed due to budget constraints.

2.1.3 "Teflonization": boosting innate antiviral defences

All living beings are constantly exposed to microorganisms that are present in the environment, and have consequently developed over time a means to cope with invasion of microbial pathogens in the body. The vertebrate immune system utilizes two general strategies to combat infectious diseases: innate and acquired immunity. Innate immunity represents the organism's first line of defence against invading pathogens. According to a concept elaborated by Janeway [53], the efficacy of the mammalian innate immune system relies on its ability to recognize pathogen-associated molecular patterns (PAMPs), or evolutionarily conserved microbial structures that are essential for the survival of the pathogens and thus cannot be changed in the course of evolution. The elicited response is not specific to a certain pathogen but directed against a group of microorganisms sharing the same microbial signature. Innate recognition of viruses, whose components are mainly all synthesized within host cells, is based on the recognition of viral nucleic acids. Discrimination between self (host) and viral nucleic acids occurs on the basis of specific chemical modifications and structural features that are unique to viral RNA and DNA, as well as on the cellular compartments where viral (but not host-derived) nucleic acids are normally found. Thus, 3 classes of PAMPs have been defined to date: dsRNA, ssRNA, and DNA containing unmethylated cytosine-phosphate-guanosine (CpG) motifs [54]. In contrast to innate immunity, acquired or adaptive immune responses are the result of a precise but rather slow process that requires, prior to the execution of effector anti-pathogen responses, the proliferation of clonally distributed immune cells expressing highly specific receptors. While the adaptive immune system can provide specific recognition of foreign antigens and immunological memory of infection, the innate immune system induces immediate immunological defence mechanisms against a large variety of pathogens. Moreover, although the duration of innate immune mechanisms is generally only of several days, its considerable contribution to the activation of more specific subsequent immune responses must not be underestimated [55].

Current knowledge concerning the molecular and cellular mechanisms linked to innate immune responses upon viral invasion in humans and in cats are reviewed in the introduction of the manuscript "The innate antiviral immune system of the cat: molecular tools for the measurement of its state of activation" presented in section 3.1 of this document, and will thus not be explained in further detail here. Particularly relevant to this project however, is the development in recent years of synthetic molecules called immune response modifiers (IRMs) that can mimic the effects of natural PAMPs and thus have been shown to induce antiviral mechanisms *in vitro* and *in vivo* in various host models. Popular IRM imitators of viral PAMPs include among others Poly IC, a synthetic double-stranded polyriboinosinic–polyribocytidylic acid, imidazoquinolines, and synthetic CpG molecules [56]. The primary consequence of stimulation of innate immune mechanisms by natural or synthetic viral PAMPs is the production of type I interferon (IFN), the family of the most biologically potent antiviral molecules known to date. Type I IFN not only represent the fundamental link between those immune cells which are first to recognize the invading viral agent, and those which confer both innate and adaptive immunity to infections [57], but also effectively induce the synthesis of various intracellular antiviral proteins which interfere with several steps of the virus replication cycle [58]. Due to the ubiquitous repartition of type I IFN receptors, expression of these effector antiviral proteins can be induced in a majority of yet uninfected cells [59]. Key components of intracellular antiviral resistance conferred by type I IFN and largely referred to in this study are the cytoplasmic Mx GTPases [60]. Initially discovered 25 years ago as indispensable for the recovery of mice from influenza virus [61], Mx proteins in various species have since been linked to defence mechanisms against many viruses. Although the mechanism of action of Mx is still incompletely understood, these intracellular proteins were found to bind to essential viral components and block their intracellular transport. In recent years, the detection of Mx is readily used as a marker for upregulation and biological activity of type I IFN [60].

The potent defence mechanisms induced by type I IFN after preventive IRM treatment could help mount a global "antiviral state", considerably restricting viral invasion and tilting the immunological balance in favour of the host. This leads to the novel concept of "teflonization" in biology. Teflon is the brand name of a synthetic polymer used to

coat cookware such as frying pans. This plastic polymer is characterized by a particularly low coefficient of friction, meaning that it is highly slippery. Consequently, most solid materials will virtually slide off any object coated with Teflon or "teflonized". With allusion to these facts, Teflon is a nickname used in media jargon given to persons, particularly politicians, to whom criticism does not seem to stick. Thus, Ronald Reagan, the 40th President of the United States, was nicknamed the Teflon President after scandals surrounding members of his staff seemed to have no effect on Reagan's popularity with the public. Along these lines, "teflonization" by an effective IRM or combination of IRMs could create a temporary shield from which viral particles would virtually "slip off" the host, thus enhancing resistance of a susceptible individual to infections by viruses. In an era of constant emergence of new viral diseases, elaboration of this idea could open to exciting new perspectives in medical science.

2.2 Objectives of the study

Four main aims were pursued in the course of this project:

1) Establishment of the necessary tools enabling to measure the expression of relevant genes during both innate and early adaptive immune responses in the domestic cat.

2) Utilization of the newly designed tools for the characterization of early immune mechanisms in the context of acute feline infections *in vitro* and *in vivo*.

3) Analysis of the immunomodulatory properties of various IRMs *in vitro* on feline cell lines and PBMCs.

4) Assessment of the antiviral or "teflonizing" (see section 2.1.3) potential of various IRMs against *in vitro* inoculation of feline cell lines with common viruses affecting the domestic cat.

2.3 Relevance of the study

A better understanding of early immune responses of the domestic cat to viral invasion, together with a methodical analysis on the possibility of manipulating antiviral defence mechanisms in order to enhance resistance to a broad spectrum of viral pathogens represents a significant step in both veterinary and human medicine. Reasons to work for the cat and reasons to work with the cat when it comes to immunological research are described in sections 2.3.1 and 2.3.2 respectively.

2.3.1 Working for the cat

Cats are affected by many dangerous viral pathogens that tend to induce latent, chronic or persistent infections with usually fatal outcomes. Due to these biological properties of feline viruses, disease management is most often long and tedious. Most protocols are based on "tender loving care" from the owner, where supportive treatment, good nursing, and patience are the key to success. Specific therapies are unavailable for most feline viral diseases, and when existent, they tend to be very costly. Moreover, in face of the ever-changing identity of feline viruses adapting to their environment, vaccines, when available, are slowly becoming insufficient for protection of domestic cats against most of these pathogens (see sections 2.1.2.1-2.1.2.6. for details on specific viruses). Thus, as feline viruses remain a danger for the domestic cat population today, the analysis of early immune responses to these pathogens could provide a better understanding of host-pathogen interactions and support the development of more effective ways to combat feline viruses. Also, prevention of viral propagation is of primordial importance in environments such as shelters, catteries and rescue homes, where many cats from different backgrounds have narrow contacts. The availability of an antiviral agent capable of transiently conferring resistance to a broad range of viruses would thus be highly desirable to temporarily protect cats when in such situations with increased risks of infection. In the development of methods and tools enabling to measure hallmarks of innate immunity, in the measurement of early immune responses to viruses in feline cells *in vitro* and *in vivo,* as well as in the screening of various IRMs for their potential to increase resistance to feline viruses *in vitro,* this study represents several steps forward in feline immunology research. Promising results could support

the development of new antiviral prophylactic drugs that would greatly contribute to the well being of cats and their owners.

2.3.2 Working with the cat

Infectious diseases have been a dominant cause of human morbidity and mortality throughout history, and emergence of new viral diseases, such as SARS, avian influenza, and Hendra encephalitis constitute a growing threat to public health (see section 2.1.1). The prospect of bioterrorist attacks represents another serious new danger that could have devastating effects. In this context, the stimulation of innate immune defences with "teflonizing" (see section 2.1.3) antiviral agents could have high relevance from both public health and economic perspectives: business travels and tourism in countries with nascent, still unidentified viruses would be of lesser worry; personnel working in hospitals, where patients affected by new viral diseases are being treated, could be more effectively protected against infection; resistance to various nosocomial infections could be conferred to patients needing elective surgery; entire populations could be temporarily protected against the spread of an "emerging" virus while awaiting the development of more specific preventive methods.

When compared to laboratory mice, the feline model holds several advantages in studies related to innate immunity and viral diseases. First, appropriate models of viral infection in mice often necessitate the modification of infectious agents or the creation of transgenic animals considerably limiting the possibilities to extrapolate data from mice to humans [62]. Second, cats belong to an outbred species, enabling more realistic consideration of individual variability in immunologic responses. Studies with felids also enable to circumvent issues related to the notable differences between the innate immune systems of mice and humans, including altered expression and repartition of TLRs [62], different NK cell activating ligands [62], and absence of important antiviral mechanisms in inbred strains [63]. Finally, while feline viruses have acquired challenging propagation strategies throughout evolution, cats have retaliated with the development of a stark innate antiviral immune system. Altogether, almost 30 subtypes of feline type I IFN have been identified [64-66] and their biological properties characterized [67, 68].

The interaction of deadly infectious agents with the cat host's genome is seen as an unprecedentedly powerful biomedical model [5]. Indeed, the domestic cat is affected by a wide range of viruses that have over time gained properties rendering their infections particularly challenging to prevent. Many natural feline viruses share biological and clinical properties with others affecting humans or other species. Along these lines, cats are commonly affected by a virus from the herpes virus family, FIV infection is considered a close model of HIV [69], and the strong antigenic and clinical diversity of feline calicivirus is similar to that of human noroviruses [70]. Even the recently discovered devastating SARS human coronavirus has a feline counterpart, FCoV. Recent events have furthermore shown that domestic cats may act as interface between humans and wild animals in the propagation of some viruses such as cowpox virus [71], rabies [72] or H5N1 avian influenza virus [73] as a result of contact with infected rodents or birds. Finally, domestic cats and their wild relative have closely witnessed emerging virus outbreaks. In the mid 1970s, a feline panleukopenia virus cultivated in a cat vaccine factory appears to have abruptly jumped from cats to dogs, producing an extremely virulent strain that caused widespread puppy mortality across the world within a few months [21]. Dogs got their chance for payback when a strain of canine distemper, endemic in the pet dogs of Masai tribesman in Tanzania, breached the species barrier and affected first hyenas and then African lions, killing a third of the huge lion population of Serengeti National Park in a six month interval in 1994 [74]. All in all, feline viral infections could prove valuable to biomedical research, providing we have a better working knowledge of the immune system of cats. Also, intervening in the host virus interactions of felids appears particularly challenging, and elaboration of efficient preventive measures by stimulation of the innate immune system in this model would reveal solid optimism for their application in humans and other animal species.

3. Publications

Two manuscripts presenting data obtained in the course of the present study were written and submitted to scientific journals. In the next sections, a brief explanation concerning the structure of text, the selection of data presented, the scientific journal in which the publication should appear, and the state of submission precedes each manuscript.

3.1 Manuscript 1

This first manuscript "*The innate antiviral immune system of the cat: molecular tools for the measurement of its state of activation* "combines many features of a review article with the presentation of original research, providing a "one-stop shop" to readers wishing to understand current state of knowledge on the innate immune system of the cat. In order to illustrate the shortcomings in the field of feline innate immunity, the Introduction gives a brief overview on innate antiviral mechanisms in humans and mice and highlights that which has been investigated in the domestic cat. This tour de force on current research in innate immunology is followed by the presentation of newly developed polymerase chain reaction (PCR) assays enabling to measure the expression of various genes selected as markers for innate immunity, as well as the utilization of these novel tools for the characterisation of early immunomodulation in feline cells following stimulation with various IRMs or inoculation with a selection of viruses *in vitro*. The validity of the assays *in vivo* is also confirmed by the measurement of an altered gene expression profile in FIV-infected kittens. All in all, this article reveals new tools for further research in feline immunity, provides new insights on host-pathogen interactions in the context of feline viral diseases, and further supports the development of the feline model for immunology research. The text has been accepted for publication in Veterinary Immunology and Immunopathology, and will appear in a special edition created following the 10th International Feline Research Retrovirus Symposium (IFRRS), which took place in May 2010.

1

The innate antiviral immune system of the cat: molecular tools for the measurement of its state of activation

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Abstract

The innate immune system plays a central role in host defence against viruses. While many studies portray mechanisms in early antiviral immune responses of humans and mice, much remains to be discovered about these mechanisms in the cat. With the objective of shedding light on early host-virus interactions in felids, we have developed 12 real-time TaqMan® qPCR systems for feline genes relevant to innate responses to viral infection, including those encoding for various IFNα and IFNω subtypes, IFNβ, intracellular antiviral factor Mx, NK cell stimulator IL-15 and effectors perforin and

granzyme B, as well as Toll-like receptors (TLRs) 3 and 8. Using these newly developed assays and others previously described, we measured the relative expression of selected markers at early time points after viral infection in vitro and in vivo. Feline embryonic fibroblasts (FEA) inoculated with feline leukemia virus (FeLV) indicated peak levels of *IFN*α, *IFN*β and *Mx* expression already 6 hours after infection. In contrast, Crandell feline kidney (CrFK) cells inoculated with feline herpes virus (FHV) responded to infection with high levels of *IFN*α and *IFN*β only after 24 hours, and no induction of *Mx* could be detected. In feline PBMCs challenged in vitro with feline immunodeficiency virus (FIV), maximal expression levels of *IFN*α, β and ω subtype genes as well as *IL-15* and *TLRs 3*, *7* and *8* were measured between 12 hours and 24 hours after infection, whereas expression levels of proinflammatory cytokine gene *IL-6* were consistently downregulated until 48 hours post inoculation. A marginal upregulation of *granzyme B* was also observed within 3 hours after infection. In an in vivo experiment, cats challenged with FIV exhibited a 2.4-fold increase in *IFN*α expression in blood 1 week post infection. We furthermore demonstrate the possibility of stimulating feline immune cells in vitro with various immune response modifiers (IRMs) already known for their immunostimulatory properties in mice and humans, namely Poly IC, Resiquimod (R-848) and dSLIM™, a synthetic oligonucleotide containing several unmethylated CpG motifs. Stimulation of feline PBMCs with dSLIM™ and R-848 effectively enhanced expression of *IFN*α within 12 hours by factors of 6 and 12 respectively, and Poly IC induced an increase in Mx mRNA expression of 28-fold. Altogether, we describe new molecular tools and their successful use for the characterization of innate immune responses against viruses in the cat and

provide evidence that feline cells can be stimulated by synthetic molecules to enhance their antiviral defence mechanisms.

Keywords: cat, innate immune system, virus, immune response modifier, real-time TaqMan® qPCR

1. Introduction

In the course of evolution, feline viruses have gained properties rendering their infections particularly challenging to prevent and treat. Presumably, the way of life of felids has driven over time the development of viruses with highly efficient viral transmission strategies and the potential to induce chronic or latent infections, thus increasing carrier populations and viral dissemination (Pontier et al., 2009). It is today widely accepted that the initial antiviral immune response of the mammalian host plays an essential role in determining the outcome of viral infection. Although extensive studies have been carried out in mouse models, the antiviral innate immune system of the cat remains poorly understood, leaving open questions regarding initial responses to viral infection as well as the possibility of manipulating innate immune mechanisms in favour of the host. In order to illustrate the current status of research concerning innate antiviral defence mechanisms in cats, we will first give a short overview of general knowledge in this field.

Studies in mice and humans have demonstrated that early pathogen recognition by the innate immune system relies on its ability to sense microbial components known as pathogen-associated molecular patterns (PAMPs) (Akira et al., 2006). With respect to

viruses, the host's intrinsic defence structures recognize mostly genomic nucleic acids and replication intermediates. Three main classes of PAMPs have emerged to date: double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and unmethylated CpG DNA. In turn, the detection of these PAMPs relies on a limited set of germline-encoded pattern recognition receptors (PRRs). Three classes of PRR molecules seem to survey various cell compartments for presence of PAMPs and promote intrinsic antiviral immunity: the Toll-like receptors (TLRs), the retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) and the cytoplasmic viral DNA sensors (Yoneyama and Fujita, 2010).

TLRs are type I integral membrane glycoproteins either expressed on the cell surface or retained in intracellular compartments. Among the 12 TLRs in mice and 10 TLRs in humans that have been identified to date (Tabeta et al., 2004), three share the ability to bind nucleic acids, namely those expressed in endosomal compartments of various subsets of dendritic cells (DCs). Thus, upon phagocytic or endocytic events, TLR3, TLR7 and TLR9 can sense viral dsRNA, ssRNA or unmethylated CpG-DNA respectively (Vaidya and Cheng, 2003). TLR8, a structurally homologous variant of TLR7, seems to be non-functional in mice, but has been described in humans as recognising similar molecular patterns to TLR7 (Jurk et al., 2002). To date, various synthetic ligands have been identified for these TLRs, among which the most popular include Poly IC, a synthetic double-stranded polyriboinosinic–polyribocytidylic acid, a well-known synthetic analogue to dsRNA and stimulator of TLR3 (Jiang et al., 2003); imidazoquinolines such as resiquimod (R-848), synthetic compounds that bind to TLR7/8 (Wagner et al., 1999); and synthetic CpG molecules, which trigger TLR9 (Vollmer et al., 2004). Such Toll-like receptor agonists have not only been used extensively for research purposes linked to the understanding of innate immune mechanisms, but have also gained much popularity in the clinical field, as treatments for

various types of disease and as vaccine adjuvants (Meyer and Stockfleth, 2008; Seya and Matsumoto, 2009).

In contrast to TLRs, RLRs and DNA sensors are ubiquitously expressed in the cytoplasm and allow infected cells to detect actively replicating virus. Two main members of the RLR family, RIG-I and melanoma differentiation associated gene 5 (MDA5), have been shown to play essential roles in the recognition of cytoplasmic viral RNA (Yoneyama et al., 2004; Loo et al., 2008). Recently, additional cytoplasmic DNA sensors have been proposed to detect viral dsDNA during infection (Yoneyama and Fujita, 2010). These molecules are currently under investigation.

PAMP-triggered PRRs activate distinct signaling pathways that converge on the activation of specific transcription factors NFκB, AP-1, IFN regulatory factor (IRF) 3 and IRF7, enhancing the expression of both proinflammatory cytokines such as IL-6 and TNFα, and type I IFNs, the main players in eradication of replicating viruses in antiviral innate immunity (Schindler et al., 2007). In humans, the large family of type I IFNs primarily include IFNα, IFNβ and IFNω (Bekisz et al., 2004). IFNα consists of a group of 13 structurally related proteins, each encoded by a separate intronless gene, while only one protein each for IFNβ and IFNω have been characterised. Type I IFNs are produced by almost all cell types, with IFNβ being the main IFN secreted by fibroblasts in response to viral challenge. Essentially, the ubiquitous presence of IFN receptor complex composed of two subunits IFNαR1 and IFNαR2 (Uze et al., 2007) and the pleiotropy of these cytokines enable IFNs to initiate potent antiviral responses. First, type I IFNs activate, in uninfected target cells, the production of intracellular effectors that can interfere with several steps of virus replication cycles. Well-studied

examples of these antiviral proteins include myxovirus-resistance protein (Mx) GTPase, the RNA-dependent protein kinase (PKR), the 2',5'-oligoadenylate synthetase (OAS) and the interferon stimulated gene (ISG) 15 (mechanisms reviewed in Sadler and Williams, 2008). Mx protein, for instance, binds to essential viral components thus blocking their intracellular transport (Haller et al., 2007). Moreover, production of this protein is widely recognized as a marker for upregulation of type I IFNs (Haller et al., 2007). Through positive feedback processes, type I IFNs were additionally shown to enhance the expression of TLRs, further sensitizing cells to microbial recognition (Siren et al., 2005). Finally, type I IFNs effectively bridge innate and adaptive immunity by promoting the differentiation and function of various immune cell populations such as DCs, NK cells, B cells, as well as CD4+ and CD8+ T cell populations (Colonna et al., 2004).

NK cells represent another important antiviral cell population (Hamerman et al., 2005). Constitutively primed to kill, these cells rapidly release IFNγ, a type II IFN affecting mainly adaptive immune cells, as well as granzyme and perforin, cytotoxic substances stored in cytosolic granules. Primary NK targets are host cells lacking MHC I molecules on their surface. As most viruses have evolved mechanisms to downregulate host cell MHC expression in order to avoid immune responses resulting from antigen presentation, virally infected cells are readily attacked by NK cells. In addition to type I IFNs, IL-15 was shown to support development and survival of NK cells as well as to stimulate their cytokine production. Liberated mainly by PAMP-activated DC, IL-15 thus also plays a crucial role in antiviral defence (Vujanovic et al., 2010).

Various essential players in the innate immune system of the cat have to date been characterised. Thus, TLRs 1-9 were shown to be differentially expressed in various feline lymphoid tissues and cell lines (Ignacio et al., 2005). Concerning TLRs 3, 7, 8 and 9, high expression levels were measured in the mesenteric lymph nodes and in the spleen. Interestingly, both B and T lymphocyte subsets purified from lymph nodes expressed TLRs and expression in feline CD4+ and CD8+ T cells was mainly restricted to these anti-viral associated TLRs. Feline viral infection in vitro also induced altered TLR expression levels in feline cells, with observed differences depending highly on cell type and TLR studied (Ignacio et al., 2005). The stimulation of feline TLRs with specific agonists has not yet been reported.

Studies carried out in the past 20 years have mainly focused on the characterisation of feline type I IFN subtypes and their potential therapeutic effects in the context of various viral diseases. Nakamura et al. cloned the first cDNA sequence for feline IFN in 1992 (Nakamura et al., 1992). The purified protein was classified as omega-type (Ueda et al., 1993b) and rapidly shown to exhibit antiviral activity both in vitro and in vivo (Tables 1 and 2). As a result, Nakamura's IFNω became the first feline antiviral drug available on the market, currently sold both in Japan (Intercat®, Toray Industries, Tokyo, Japan) and Europe (Virbagen®omega, Laboratoire Virbac, Carros Cedex, France) to treat feline calicivirus and canine parvovirus infections. Subsequently, therapeutic effects of this product as stand-alone or combinatorial agent were described in the context of a series of feline and canine diseases (Table 2). In recent years, many feline type I IFN subtype nucleic acid and amino acid sequences have been characterized. In all, 13 subtypes each of the *feIFNα* and *feIFNω* genes have been cloned and the biological antiviral properties of the purified proteins were demonstrated

in vitro (Table 1). Studies concerning the structure of these IFNα and IFNω subtypes have indicated that both protein families have high homology and that feline IFNω is more similar to the IFNα than to the IFNω of other animal species. Although the individual functions of all these IFN subtypes are unknown in the cat, the impressive number of *type I IFN* genes identified to date underlines the necessity of broad antiviral responses in felids.

Regarding effector functions of feline type I IFNs, mainly the induction of intracellular antiviral proteins has been discussed. In this way, the biological activity of Nakamura's recombinant feline IFN was initially supported by its potential to modulate OAS activity in vitro (Ueda et al., 1993a). The physiological effects of oral or ocular treatment with this recombinant IFNω were later assessed by local and systemic measurement of the feline Mx protein (Bracklein et al., 2006), which had already been previously described (Horisberger et al., 1990). More recently, recombinant IFNω stimulation of feline cells was shown to induce expression of feline ISG15. Molecular cloning of this gene enabled detection of the immunomodulatory properties of both cell-conjugated and free forms of this antiviral effector protein (Tanabe et al., 2008). Altogether, these studies illustrate the breadth of IFN-induced antiviral mechanisms in the cat. The recent cloning and molecular characterisation of the feline IFNαR2 (Xue et al., 2010) may support the study of further effector functions of type I IFNs in the cat.

Finally, only few studies have targeted aspects of the feline innate immune response following viral encounter. Dean et al. reported that immunodeficiency in FIV infection also concerned early anti-pathogen defence mechanisms in vivo (Dean et al., 1998). Thus, chronically FIV-infected cats mounted weaker initial immune responses against *Listeria monocytogenes*, leading to a more severe form of infection. In a further study,

inoculation of FIV-infected cats with modified *L.monocytogenes* carrying an expression vector for various feline cytokines indicated that IL-15 played a central role in restoring FIV-related innate immune disturbances and increasing the natural killer cell population (Dean et al., 2006).

Despite extensive studies on molecular structure and antiviral effects of feline IFN, much remains to be understood regarding qualitative, quantitative and timely aspects of innate immune mechanisms following viral infection in the cat. Moreover, the possibility of synthetically stimulating the intrinsic immune system has not yet been investigated in this species.

Herein, we describe the development of twelve real-time qPCR assays enabling us to quantify the expression of key factors involved in feline innate immunity. Relative expression levels of various innate immune parameters were determined in viral infection both in vitro and in vivo. Furthermore, we report the quantification of innate immune responses obtained in vitro after stimulation of feline immune cells with various synthetic molecules.

2. Materials and Methods

2.1 Cats, FIV infection and blood collection

Ten male specified pathogen-free (SPF) cats of 10 weeks of age and 4 male spf cats of 4 years of age from Liberty Research Inc. (Waverly, NY, USA) were used in this study. Young and adult cats were housed separately in an animal-friendly environment and under optimal ethological conditions. All experimental procedures were reviewed and approved by the Swiss Federal Veterinary Office.

After an adaptation phase of 4 weeks, the kittens were infected intraperitoneally with 50 cat infectious doses 50 (50 CID_{50}) of the FIV Glasgow 8 (GL8) strain, previously titrated *in vivo* and kindly provided by Dr. M. Hosie and Prof. O. Jarret from the University of Glasgow, Great Britain. Whole blood was collected in EDTA-supplemented evacuated tubes both on day 0 before infection and on day 7 post infection. 100µl of blood was mixed with 300µl mRNA lysis buffer (Roche Diagnostics, Rotkreuz, Switzerland) immediately after blood collection and samples were stored at -80°C until further analysis.

EDTA-supplemented venous blood from the 4 adult spf cats was used for the purification of PBMCs required for other experiments, namely real-time qPCR assay optimisation, IRM stimulation or inoculation with FIV.

2.2 Feline PBMC isolation, cell lines and cell culture

Feline PBMCs were isolated from EDTA-supplemented whole blood by Ficoll-Hypaque density gradient centrifugation (Histopaque®-1077, Sigma-Aldrich, Buchs, Switzerland). The mononuclear cell fraction was washed once and resuspended in RPMI with Glutamax I (Gibco®, Invitrogen, San Diego, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (Bioconcept, Allschwil, Switzerland), 100 U/ml penicillin and 100µg/ml streptomycin (Gibco®, Invitrogen, San Diego, CA, USA). For the production of cDNA for real-time qPCR assay optimization, isolated PBMCs were either stimulated directly after isolation with 4µg/ml LPS (Sigma-Aldrich, Buchs, Switzerland) or with a combination of 10µg/ml Concavalin A (Sigma-Aldrich, Buchs, Switzerland) directly after isolation and 50U/ml IL-2 (Sandoz Pharmaceuticals AG, Cham, Switzerland) 24h and 96h post isolation. For experiments including IRM

stimulation or FIV inoculation of feline PBMCs, the isolated cells were counted using the Sysmex XT 2000 iV (Sysmex, Norderstedt, Germany) as previously described (Weissenbacher et al., 2010) and dispersed in 96-well plates at a density of $3x10^5$ cells per well in 100µl complete RPMI.

Crandell-Reese feline kidney (CrFK) cells and Feline Embryonic Fibroblasts (FEA) were maintained in RPMI supplemented as described above.

2.3 Viruses and in vitro inoculation experiments

FIV GL8 and FeLV-A/Glasgow-1 strains, generous gifts from Dr. M. Hosie and Prof. O. Jarret (University of Glasgow, Great Britain), had been previously propagated and titrated on purified feline PBMCs and FEA cells respectively. The FHV ZH5-04 strain was kindly provided by Veterinaria AG (Zurich, Switzerland), had undergone several passages in CrFK cells and was titrated in these cells before use in the present study. All viruses had been kept at -80°C for long-term storage.

For in vitro inoculation experiments of adherent cells, $2x10^4$ FEA or CrFK cells were seeded in the wells of a 96-well plate and incubated at 37°C, 5% CO_2 for 24 hours. FEA cells were then inoculated with 20 tissue culture infectious dose 50 ($TCID_{50}$) FeLV and CrFK cells with 50 $TCID_{50}$ of FHV. For in vitro inoculation of feline PBMCs, $3x10^5$ cells isolated from one individual adult cat were distributed in wells of a 96-well plate and directly infected with 50 $TCID_{50}$ of FIV stock virus. The cultures were incubated at 37°C, 5% CO_2 and cells from duplicate wells were harvested at the time intervals depicted in the results of the respective experiments. For each time point, duplicate wells containing uninfected cells were included as unstimulated controls for comparison in gene expression profiles. For harvesting of adherent cells, the supernatant was removed from each well and the cells were directly lysed with 300µl mRNA lysis buffer

(mRNA isolation kit I, Roche Diagnostics). PBMCs from separate wells were first pelleted and subsequently lysed in the same manner. Lysed samples were stored at -80°C until further analysis. In experiments with FHV and FeLV, the presence of virus-specific nucleic acids in supernatants of infected cells was confirmed by real-time qPCR 24 and 48 hours post inoculation respectively using systems previously published (Vogtlin et al., 2002; Tandon et al., 2005). Measurement of FIV provirus integration in PBMCs was carried out by real time PCR as has already been described (Leutenegger et al., 1999).

2.4 IRM stimulation of feline PBMCs

Purified PBMCs from one adult cat were counted and $3x10^5$ cells per well were seeded directly after isolation in a 96-well format. Cells were treated with either 144µg/ml dSLIM™ (Mologen AG, Berlin), 20µg/ml R-848 or 20µg/ml Poly IC (Alexis biochemicals, Enzo Life Sciences AG, Lausen, Switzerland) and maintained at 37°C, 5% CO_2. dSLIM™ and Poly IC were solubilized in PBS, R-848 in DMSO, as recommended by the manufacturers. All solutions were diluted in PBS so that cell treatments were equi-volume. Two controls for comparison in gene expression profiles were added in which cells were treated with either an equal volume of PBS, or, for comparison with R-848 stimulation, PBS containing the corresponding concentration of DMSO. After 6, 12, 24 and 48 hours, cells were harvested as described above for PBMCs.

2.5 RNA isolation and synthesis of cDNA

For real-time qPCR assay optimization experiments, total RNA was extracted manually from pellets of $2.5x10^6$ PBMCs using the RNeasy®Plus Mini Kit (Qiagen AG, Hilden, Germany). Cell lysis and homogenization was thereby carried out using the QIAshredder™ and genomic DNA (gDNA) was removed with the gDNA Eliminator spin column (Qiagen AG) according to the manufacturer's recommendations. For experiments concerning the measurements of cytokine gene expression, mRNA extractions were performed with the mRNA Isolation Kit I and MagNA Pure LC Instrument (Roche Diagnostics) according to the manufacturer's instructions. In both cases, purified RNA was stored at -80°C until further use.

First strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions, and samples were stored at -20°C until use for qPCR measurements.

2.6 Real-time qPCR

2.6.1 General conditions

Real-time qPCR was carried out using a Rotor-Gene 6000 real-time rotary analyser (Corbett, Mortlake, Australia). PCR assays comprised 5µl of cDNA in a total volume of 25 µl per reaction using the TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). Thermocycling conditions consisted of an initial denaturation of 20s at 95°C, followed by 45 cycles of amplification by melting at 95°C for 3s and annealing at 60°C for 45s. Systems for *TLR 7* and *TLR9* were used as previously described (Ignacio et al., 2005).

2.6.2 Development and validation of novel assays

Primers and probes for each gene listed in Table 1 were designed using Primer Express™ software (versions 2 and 3, Applied Biosystems). The sequences were retrieved from Ensembl (http://www.ensemble.org) and GeneBank (http://www.ncbi.nlm.nih.gov) and oligonucleotides were commercially synthesized (Microsynth, Balgach, Switzerland). All probes were labelled with the reporter dye FAM (6-carboxyfluorescein) at the 5' end and the quencher dye TAMRA (6-carboxytetramethylrhodamine) at the 3'end. For genes containing introns, the designed systems covered putative exon-exon junctions, in order to preclude genomic DNA amplification. Primers for each system were first tested for the generation of amplicons of expected length in PCR assays comprising cDNA synthesized from ConA-stimulated PBMCs. The PCR products were subjected to gel electrophoresis on 3% agarose gels, stained with ethidium bromide and visualized using the Chemigenius 2 BioImaging System (Syngene, Cambridge, UK). Only primers indicating appropriate specificity and amplification products of correct length were utilized for further optimisation. Primer and probe concentrations for each system were optimized on five-fold serial dilutions of cDNA derived from ConA-stimulated PBMCs. cDNA was diluted in 30µg/ml salmon sperm DNA (Invitrogen, Basel, Switzerland) and quadruplicates of each dilution were tested simultaneously. Matched forward and reverse primer concentrations of 300, 600 and 900nM were evaluated first with a probe end concentration of 250nM. The performance of the assays was further optimized using the best primer concentrations combined with 3 different probe end concentrations, namely 50, 150, and 250nM. Amplification efficiencies of the newly designed assays were calculated as previously described (Klein et al., 1999) using the following equation:

$E = [10^{(-1/slope)}]-1.$

The system for *IL-6* had already been described (Taglinger et al., 2008). Optimization experiments for this assay in our laboratory indicated best results with concentrations of 800nM for forward and reverse primers and 250nM for the probe, rendering an efficiency of 0.99. We also designed new systems for *TLR 3* and *TLR8*, as we were unable to obtain an adequate efficiency with those previously described (Ignacio et al., 2005).

2.6.3 Relative expression analysis of feline genes

Expression levels of selected genes were calculated using GeNorm version 3.5 (Vandesompele et al., 2002), using *β-glucuronidase* (*GUSB*) and *tryptophan 5-monooxygenase activation protein, zeta polypeptide* (*YWHAZ*) as reference genes, under conditions previously validated for the feline species (Kessler et al., 2009). For illustration of relative expressions of the various genes in the figures of the present report, a cut-off of 2-fold up- respectively downregulation measured at least at one time point in each experiment was selected.

2.7 Statistical analysis

For the measurement of the effect of FIV on cytokine expression in vivo, statistical analysis was performed with GraphPad Prism for Windows, Version 3.0 (GraphPad software, San Diego, CA, USA). Normalized cytokine expression factors were tested for statistical differences between samples of week 0 and week 1 post infection using a non-parametric Wilcoxon signed rank test for paired samples.

3. Results

3.1 Development of novel real-time qPCR systems

Primer and probe sequences for real-time qPCR systems of 12 feline genes relevant to innate immune responses against viruses were designed and validated (Table 1). The assay developed for *IFNω* encompasses the expression of all *IFNω* subtype genes simultaneously. Due to the sequence dissimilarities in *IFNα* subtype genes, several systems had to be created for this *type I IFN* family: a separate assay was designed for *IFNα14* while common probes and reverse primers are shared by the *IFNα3*, *IFNα7* and IFNα systems that recognize *IFNα subtypes 3, 7* and the remaining subtypes (*IFNα1, 2, 5, 6, 8-13*) respectively.

Amplicon sizes of the developed real-time qPCR assays ranged from 69 to 135 bp. A single band could be visualized after initial amplification analysis of primers only, when tested with cDNA derived from purified feline PBMCs. Both primer and probe concentrations were optimized and reaction efficiencies were evaluated.

3.2 Stimulation of feline cells with various IRMs

Both immortalized and primary cells of feline origin were stimulated with 3 IRMs of different classes, namely dSLIM™, a covalently-closed oligonucleotide containing several unmethylated CpG motifs (Schmidt et al., 2006), R-848 and Poly IC. The concentrations indicating optimal IFNα induction after 12 hours in preliminary titration experiments were selected for use in this study (data not shown). As determined by both trypan blue exclusion and cell count measurements at regular intervals after stimulation, no evidence of cellular toxicity could be observed in CrFK cells, FEAs or purified feline PBMCs treated with dSLIM™ or R848 (data not shown). Poly IC, however, at

concentrations capable of inducing innate immune responses, induced cell death in all three cell types, and housekeeping gene expression analysis in cell samples treated with this molecule repeatedly indicated an increase of 6-8 Ct values compared to untreated controls.

IRM-treated cells were systematically screened for increased expression of *IFNα*, *Mx* and *IL-6* as indicators of successful stimulation. Despite measurable basal expression levels of *TLRs 3*, *7*, *8* and *9* in CrFK and FEA cells, all 3 IRMs failed to influence the transcription of genes relevant to innate immunity (data not shown). However, differential gene profiles were observed after treatment of purified feline PBMCs with the immune-stimulating molecules (Figure 1A-C). dSLIM™ and R-848 induced a 6- and 12-fold upregulation of *IFNα* expression within 12 hours of stimulation. However, no increase in transcription of *Mx* could be measured in PBMCs treated with these two molecules. In cells pulsed with Poly IC, levels of *IFNα* expression could not be accurately measured due to both low basal expression levels of this cytokine and cell death induced by this IRM. Nevertheless, Poly IC induced a 28-fold increase in *Mx* expression that could be measured already 6 hours post stimulation. Upregulation of *IFNα* expression was concordant with that of *IL-6* after stimulation of PBMCs with Poly IC and R-848. In contrast, treatment of these cells with dSLIM™ induced significant downregulation of *IL-6* expression. Finally, among all 3 IRMs, only dSLIM™ was found to modulate *TLR* expression, indicating a 6-fold increase of *TLR9* within 12 hours of stimulation.

3.3 Early immune responses of feline cell lines after inoculation with feline viruses

The induction of host cell immune responses by FHV and FeLV were assessed in vitro on CrFK cells and FEAs respectively at regular time intervals early post infection. Productive infection of the cells was confirmed by real-time PCR of cell culture supernatants 24 and 48 hours post inoculation for FHV and FeLV respectively (data not shown). Modulations in relative expressions of *IFNα, IFNβ, Mx* and *IL-6* were utilized as parameters for immune responses by the cells. Progressively increasing levels of *IFNα, IFNβ* and *IL-6* were measured after inoculation of CrFK cells with FHV, with highest levels corresponding to respectively 37-, 45- and 23-fold upregulation in gene expression measured after 24 hours. No changes in *Mx* expression were observed (Figure 2A). In contrast, FEA cells indicated the most potent antiviral immune response just 6 hours after inoculation with FeLV, and transcription levels of all analysed genes decreased progressively thereafter. Within 6 hours post inoculation, increases of 15-fold in *IFNα* expression, 29-fold in *IFNβ* expression and 4-fold in *IL-6* expression were measured. Presence of *Mx* mRNA was proportional to levels of *IFNα* and *IFNβ* expression, with highest levels also noted 6h post inoculation (Figure 2B).

3.4 Evaluation of innate immune response parameters in feline PBMCs after in vitro infection with FIV

The influence on the expression of 14 genes including receptors and cytokines relevant to innate immunity was tested in feline PBMCs at regular time points after inoculation with FIV. Effective integration of FIV provirus was confirmed by real-time PCR 48 hours after infection of the cells (data not shown). Modulations in the expression of the tested factors are depicted in Figure 3. All genes tested were influenced within the first 48 hours of stimulation, with the exception of *TLR9* (not shown). The strongest

response was measured 12 hours after infection, with substantial modulation at this time point in mRNA levels of 9 out of 10 cytokines tested. *Type I IFN* genes were upregulated as of 6h post inoculation. Peak levels of 374-fold and 121-fold inductions in gene expression of *IFNα* and *IFNω* were achieved at 12 hours post infection, while strongest induction of *IFNβ*, namely by a factor of 114, was measured 24 hours after FIV inoculation. Although maximal gene expression levels were slightly lower for the individual *IFNα* subtypes tested (65-fold, 67-fold and 24-fold for *IFNs 3*, *7* and *14* respectively), a similar pattern of stimulation was observed, with highest expression 12 hours after infection. *Mx* and *IL-15* genes were induced in a manner proportional to the *type I IFN* genes at all time points measured with highest increases at 12 hours post inoculation of 38-fold and 7-fold respectively. During the first 48 hours, FIV infection downregulated *IL-6* expression attaining lowest levels within 6 hours after inoculation, when a 60-fold decrease in expression was observed. Genes encoding for contents of NK cell cytotoxic granules appeared only marginally affected when their expression was measured in PBMCs. Both *perforin* and *granzyme B* mRNA levels were maximally increased 3h post infection by 1.8-fold and 2-fold respectively. Similarly, the expression of *TLR* genes in PBMCs was only slightly influenced by FIV infection. Transcription of *TLRs 3*, *7* and *8* was however induced in a delayed manner when compared to the cytokines, attaining maximal levels 24 hours after infection with induction factors ranging from 2 to 2.6.

3.5 Measurement of type I IFN in cats after FIV challenge in vivo

10 spf cats were subjected to FIV infection with the highly virulent GL8 strain. Expression levels of feline *IFNα* were compared in whole blood samples collected from the cats immediately before and 1 week after FIV challenge infection. 9 out of 10 cats

displayed higher *IFNα* expression after infection. An average of 2.4-fold induction in expression of this cytokine could be measured in the cats after infection (p=0.0059) (Figure 4).

4. Discussion

Innate immune responses to invading pathogens play a key role in the outcome of infection. Over the course of time, mammalian hosts have developed the capacity to counterbalance virus attacks through sensing of viral signatures by specialized immune cells and immediate activation of the type I IFN system. Within minutes to hours, this family of antiviral cytokines initiates an explosion of potent defence mechanisms, leading to the suppression of viral replication and protection of the host. These intrinsic immune responses remain poorly understood in felids, a species that seems particularly sensitive to viral infections, with sporadic viral outbreaks compromising endangered populations (Evermann et al., 1988; Roelke-Parker et al., 1996; Cunningham et al., 2008; Meli et al., 2010). Until now, the appropriate tools for characterizing innate immune mechanisms in this species were unavailable. In order to better understand the early host-pathogen interactions occurring in feline viral infections, we describe herein the development of 12 real-time qPCR systems to measure the expression of feline genes related to innate antiviral defence mechanisms. Through a series of experiments, we demonstrate the possibility of monitoring key events in innate immune responses both in vitro and in vivo. We further demonstrate the initiation of antiviral responses in feline immune cells upon stimulation with various IRMs.

The strength, extent and kinetics of immune responses elaborated by feline cells upon viral inoculation in vitro were evaluated. In these experiments, expression levels of *IFNα* and *IL-6* were systematically analyzed as indicators for the initiation of innate immune mechanisms. As *Mx* expression is strictly regulated by type I IFN (Holzinger et al., 2007), biological functionality of the induced immune responses was assessed by simultaneous analysis of this factor. In all models of infection used in this study, we observed the characteristic transient expression of *type I IFN* genes, with a duration ranging from 6 to 12 hours in CrFK cells and FEAs inoculated with FHV and FeLV respectively to 36 hours in PBMCs infected with FIV. The presence of specialized cells for the production of type I IFNs in the blood is most likely linked to the longer expression of these innate cytokines in PBMCs (Colonna et al., 2004). Moreover, *IFNs* *α, β* and *ω* were expressed at differential levels in feline cells upon infection; while PBMCs heavily upregulated expression of *IFNα* and *IFNω* genes, cell lines retaining epithelial and fibroblast properties preferentially increased production of *IFNβ* mRNA levels. Time points at which highest expression of antiviral genes was measured also greatly varied with cell type and virus. Although FEA cells responded to presence of FeLV within 6 hours, induction of innate immune gene expression could only be observed after 24 hours following FHV infection in CrFK cells. Peak antiviral response in PBMCs was measured 12 hours after infection, with evidence for progressive development and rapid decrease of the response. Further experiments are necessary to determine the significance of individual properties of cells and tissues as well as virus-related factors such as source and infectious dose in these observations.

Interestingly, although high levels of *type I IFN* expression were observed 24 hours after FHV infection of CrFK cells, no induction of *Mx* was measured, indicating that this virus may have evolved mechanisms to interrupt IFN-induced host defence

mechanisms in infected cells. Indeed, many viruses have evolutionarily acquired sophisticated strategies to counteract the IFN system (Katze et al., 2002). Various members of the herpes virus family have been reported to directly inhibit the proteins that mediate the antiviral state (Elia et al., 1996), interfere with the expression of signaling molecules downstream of the IFNαR (Miller et al., 1998), or hypothetically support the disassembly of nuclear structures to get rid of antiviral components (Van Sant et al., 2001). These mechanisms have been linked to persistence of infections induced by some viruses of this family. Although there is no evidence for a specific viral inhibitor of Mx proteins so far, viruses can subvert the Mx system through exceptionally fast growth in host cells (Haller et al., 2007) or by affecting signal transduction upstream of IFN-regulated gene transcription. The presence of other antiviral factors in FHV-infected feline cells could shed light on possible mechanisms linked to these findings.

FIV can readily infect feline immune cells in vitro, offering a system that enables the study of innate antiviral immune reactions in a heterogeneous population of cells that actively take part in complex networks of immune interactions both in vitro and in vivo. Consequently, we studied the effect of viral inoculation in PBMCs on the expression of all cytokines and receptors for which we had developed real-time qPCR systems. High levels of *type I IFN* expression were measured in response to infection, again most probably linked to the activation of professional dendritic cells producing extraordinary amounts of these cytokines (Colonna et al., 2004). Similar expression patterns were noted for all *IFNα* gene subtypes analysed. Lower induction rates of *IFNα3*, *7* and *14* subtypes are most likely due to the measurement of individual genes versus the simultaneous quantification of many subtypes with the real-time assay developed for the

remaining *IFNα* subtypes. Although the induction of *type I IFN* genes largely overlap, the array of IFNα and IFNω subtypes produced upon stimulation seems to be determined by cell-specific levels of transcription factors IRF3 and IRF7 (Sato et al., 2000). Furthermore, the extent of biological activity conferred by each individual protein depends on a variety of factors. Thus, the differential affinity of each subtype to the subunits of the IFNαR, the surface expression level of IFNαR1 and IFNαR2 in target cells, as well as the lifetime and stability of the ligand–receptor complex all play a role in the extent of the host response to infection (Genin et al., 2009). Different pathogens may influence, in this way, the phenotype of the developing immune response by shaping the profile of IFN subtypes induced (Foster et al., 2004). As previously shown for the human IFN system (Loseke et al., 2003), the expression analysis of individual subtypes by real-time qPCR can provide valuable information on host innate immune responses to pathogens. We have initiated the development of appropriate tools to study these mechanisms in more detail in feline species.

Infection of feline PBMCs with FIV led to a substantial downregulation of the expression of the proinflammatory cytokine gene *IL-6*. These findings are reminiscent of previous experiments in which a 100 to100,000-fold reduction of *IL-6* transcription was observed in monocytes from FIV-infected cats (Kipar et al., 2004). Downregulation of this cytokine was reported to be more intense when infection of the monocyte fraction itself could not be demonstrated (Kipar et al., 2004). Hypothetically, factors liberated by primarily infected lymphocytes could affect function and immune responses of monocytic cells, the main producers of IL-6 in blood. Thus, further investigations on PBMC subpopulations are required to determine the role of preferential cell tropism of certain viral strains on measured cytokine responses.

Our results further indicate a type I IFN-regulated pattern of NK cell stimulator IL-15 gene expression, supporting the previously described role of IFNs in stimulation of NK cell activity (Vujanovic et al., 2010). In contrast, effectors of NK cytotoxicity were only marginally induced as measured in PBMCs acutely infected with FIV in this study. Although low levels of increase in *perforin* and *granzyme B* mRNA production have been described upon stark stimulation, preformed transcripts for these proteins have been detected in resting NK cells (Fehniger et al., 2007). Gene expression analysis in separate immune cell populations as well as specific cytotoxicity assays would confer additional information on the biological value of our observations for these NK cell related cytokines.

Analysis of the expression of TLR genes demonstrated slight enhancement in mRNA levels of *TLRs 3, 7* and *8* during the peak time point of innate immune response against FIV. The ssRNA phenotype of this virus coupled with the presence of dsRNA after reverse transcription during intracellular replication support the requirement for selectively higher levels of these TLRs upon infection. The relatively low levels of induction observed could be explained by the presence of a pool of receptors in the endoplasmatic reticulum that is actively transported to the endosomes following stimulation of the cell (Latz et al., 2004). Although upregulation of *TLR9* after FIV inoculation of CrFK cells has been reported (Ignacio et al., 2005), our observations regarding mRNA expression of this *TLR* gene in acutely FIV-infected PBMCs do not corroborate with the results previously published.

To our knowledge, this is the first report showing successful stimulation of innate mechanisms in feline immune cells by various IRMs. Both kinetics and potency of responses could be measured. Since mRNA expression of proinflammatory cytokine

gene *IL-6* had been previously used as readout for stimulation through TLRs in pancreatic islet cells (Franchini et al., 2010), we included this cytokine as comparative indicator for biological activity of TLR agonist. Our results indicate that *IFNα* is a more reliable marker for TLR stimulation in immune cells due to higher stability in expression over time and apparent higher expression of these cytokines.

Both R-848 and dSLIM™ could efficiently enhance *IFNα* expression within 12 hours. This level of stimulation was likely insufficient to induce measurable increase in *Mx* expression, as induction of the gene encoding for this antiviral factor was repeatedly 5 to 10 times lower than that of IFNα in our experiments.

dSLIM™ is a non-coding DNA molecule that contains several unmethylated CpG motifs and possesses the broad spectrum immunomodulatory properties of Class C CpG ODNs (Schmidt et al., 2006). In vitro and in vivo studies have demonstrated immunomodulatory effects and safety of dSLIM™ both in humans and mice (Wittig et al., 2001; Kochling et al., 2003). Moreover, this molecule has exhibited protective immune stimulation in human colon cancer and is currently undergoing second phase clinical trials (Weihrauch et al., 2005). In addition to positively influencing *IFNα* expression in feline immune cells, dSLIM™ was the only tested IRM that affected *TLR* mRNA levels, leading to a 6-fold upregulation of *TLR9*. Further experiments will determine whether such properties could support combinatorial use of this molecule to enhance effects of other IRMs.

Poly IC was toxic to feline cells already at the lowest concentrations capable of inducing innate immune responses. Toxicity of this product has long been acknowledged and studied both in vitro and in vivo (Homan et al., 1972; Lv and Bao, 2009). Mechanisms by which cell death is induced remain unclear, however it has been

suggested that pathways leading to the induction of *type I IFN* genes are uncoupled from apoptotic pathways (Han et al., 2004). We measured high induction of *Mx* in Poly IC-treated PBMCs after just 6 hours post inoculation. While the possibility that Mx proteins fulfil a cellular function involved in cellular trafficking and/or in stress responses has been postulated (Horisberger, 1992) it remains more likely that presence of this antiviral factor is indicative of IFNα function.

Finally, the experiments here described were carried out with PBMCs from only one cat in order to ensure comparability of both the effects of infection and IRMs at different time points and the relation between expression levels of the cytokines tested. Admittedly, innate immune responses may vary considerably between individuals of an outbred species; however, when purified PBMCs from 8 adult SPF cats were previously utilized in similar smaller experiments, only slight variation was observed in their response to IRM stimulation and FIV infection (data not shown). Studies with cells from cats in the field would most likely give relevant information about individuality in responses after IRM stimulation or infection.

Altogether, we describe the development of tools to measure antiviral innate immune responses in the cat and show their successful utilization in the context of various virus infections in vitro and in vivo. Moreover, we provide initial evidence of the possibility of inducing innate immune responses in feline immune cells by stimulation with various IRMs. Future plans include screening of a vast array of IRMs for optimal manipulation of the feline innate immune system and enhancing the resistance of felids to viral infection.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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856

Table 1: Cloned feline *type I IFN* subtypes and the antiviral activity of their purified proteins in vitro

Feline type I IFN genes			In vitro antiviral activity of purified proteins		
Subtype	**Cell type / tissue for cloning**	**Reference**	**Virus**	**Cell line**	**Reference**
IFNω [1,2]	T-cell line (LSA-1)	Nakamura et al., 1992	FCV	CrFK	Sakurai et al., 1992
			VSV, FCV, FHV, FCoV, FPV, rotavirus	CrFK, fcwf-4, MDCK	Mochizuki et al., 1994
			FIV	FetJ-Bang	Tanabe and Yamamoto, 2001
IFNα1-3, 5, 6	Mesenteric lymph node cells	Wonderling et al., 2002	VSV, FCV	CrFK, AH927	Baldwin et al., 2004
IFNα7-14	Epithelial cell line (CrFK)	Nagai et al., 2004	VSV	CrFK, fcwf-4, RK-13, MDBK, MDCK, L-929, FL	Taira et al., 2005
IFNω1-13	Spleen of FCoV-infected cat	Yang et al., 2007	VSV	CrFK, MDCK, MBDK	Yang et al., 2007

1 initially classified as IFNω, re-termed "ω-like" by Yang et. al, 2007
2 purified protein commercialized and utilized in various *in vivo* studies (see text and Table2)
VSV: vesicular stomatitis virus, FCV: feline calicivirus, FHV: feline herpes virus, FCoV: feline coronavirus, FPV: feline parvovirus, FIV: feline immunodeficiency virus, AH927: feline embryo fibroblast cell line, CrFK: Crandell feline kidney cells, fcwf-4: felis catus whole fetus cells, FetJ-Bang: persistently FIV-infected feline T-cell line, FL: transformed human amnion cells, L-929: mouse fibroblast cell line, LSA-1: cells derived from thymic lymphosarkoma of feline leukemia virus positive cat, MDBK: Madin-Darby bovine kidney cells, MDCK: Madin-Darby canine cells, RK-13: rabbit kidney-derived cells

856

856 **Table 2**: In vivo studies on antiviral effects of feline IFNω (Intercat® and Virbagen®omega)

Disease	Reference
CPV infection	Minagawa et al., 1999 Martin et al., 2002
FeLV infection FeLV/FIV co-infection	de Mari et al., 2004
FeLV infection	Collado et al., 2007
FPV infection	Paltrinieri et al., 2007
FHV infection	Haid et al., 2007
Herpes dermatitis	Gutzwiller et al., 2007
Chronic gingivostomatitis	Southernden and Gorrel, 2007
FCV	Ohe et al., 2008

CPV: canine parvovirus, FCV: feline calicivirus, FeLV: feline leukemia virus, FHV: feline herpes virus, FIV: feline immunodeficiency virus, FPV: feline parvovirus

Table 3: Real-time qPCR systems for 12 feline genes related to innate immunity

Gene	Accession Number	Oligo	Sequence	Final conc. (nM)	Amplicon size (bp)	Efficiency
IFNα	AY117395[1]	Forward	CACGTGACGAACCAGAAGATCTT	300		
		Probe	ACTTCTTCTGCACAGAGGCGTCCTCG	300	74	1.04
		Reverse	GAGGGTGGTGTTCCAAGCA	250		
IFNα3	AY117393[1]	Forward	CGTGACGAACCAGGAGATCTTC	900		
		Probe	ACTTCTTCTGCACAGAGGCGTCCTCG	900	72	1.07
		Reverse	GAGGGTGGTGTTCCAAGCA	250		
IFNα7	AB094996[1]	Forward	CACGTGACCAACCAGAAGATCTT	600		
		Probe	ACTTCTTCTGCACAGAGGCGTCCTCG	600	74	1.13
		Reverse	GAGGGTGGTGTTCCAAGCA	150		
IFNα14	AB095003[1]	Forward	CGTCTGCTCTCTGGGTTGTG	600		
		Probe	CCTGCCTCAGACCCACGGCC	600	77	1.00
		Reverse	ATTTGTCCCAGGAGCGTCAA	150		
IFNβ	AB021707[1]	Forward	TGGAATGAGACCACTGTTGAGAA	900		
		Probe	CTCCTTGCGACACTCCACTGGCAG	900	69	0.97
		Reverse	GGATCGTTTCCAGGTGTTCCT	50		
IFNω	DQ420220[1]	Forward	CGCAGGTTAGCAGGGACAAC	600		
		Probe	CGGAGACTGTCCCCTTTCTTGTGCC	600	93	1.07
		Reverse	GGGAAGCGGAAGTCTTTTCTG	50		
Mx	NM002462[1]	Forward	ACCAGAGCTCGGGCAAGAG	900		
		Probe	CCTTCCCAGAGGCAGCGGTATTGTC	900	96	1.00
		Reverse	TTCAGCACCAGAGGACACCTT	250		
IL-15	ENSFCAG00000011861[2]	Forward	AGTGATGTTCATCCCAATTGCA	400		
		Probe	TTCGCTTGAGTCCAAAAATGCGACCA	400	135	1.00
		Reverse	ACCGCTGTTTGCTAGGATAATAATG	50		
TLR3	ENSFCAT00000006197[2]	Forward	CAACAACTTAGCACGGCTATGG	400		
		Probe	AACGTGCAAACCCTAGTGGTCCTGTTGATT	400	72	1.07
		Reverse	AATGTGGAGGTGAGAAAGACCC	80		
TLR8	ENSFCAG00000007243[2]	Forward	GCTCCAGCTGTTTCCTCATC	400		
		Probe	CCAGTTGCTCGACTTAAGTGG	400	82	1.01
		Reverse	GAGGCTGTTGGTCAAAGAGG	80		
Perforin	EU032539[1]	Forward	TTCGCGGCCCAGAAGAC	900		
		Probe	TTCCACGACCAGTACAGCTTCAGCACTG	900	79	0.98
		Reverse	GTGAGAGCTGTAGAAGCGACATTC	250		
Granzyme B	EU153367[1]	Forward	CCACCCAGACTATAATCCAAAGAA	600		
		Probe	CCAACGACATCATGTTACTGCAGCTGG	600	77	0.97
		Reverse	CAGTCAGCTTGGCCTTTTTCA	250		

1 GenBank, 2 Ensembl (http://www.ensembl.org/index.html)

856

Figure Legends

Figure 1. Kinetics of innate cytokine expression in feline PBMCs after IRM stimulation. Feline PBMCs of one cat were incubated for 6, 12, 24 and 48 hours with A) 144µg/ml dSLIM™, B) 20µg/ml R-848 and C) 20µg/ml Poly IC. mRNA expression of the indicated genes was measured by real-time qPCR and normalized to expression of two feline housekeeping genes (*GUSB* and *YWHAZ*). Depicted expression factors at each time point represent the ratio of measured mRNA levels in the IRM-stimulated samples compared to samples treated with a negative control. Only genes indicating at least a 2-fold modulation in their expression at any time point are shown. Data represent means of duplicate reactions for each time point. Standard deviations are shown as an indication for reproducibility.

Figure 2. Innate immune responses to FHV and FeLV in vitro. A) CrFK cells were inoculated with 50 $TCID_{50}$ of FHV and B) FEA cells were inoculated with 20 $TCID_{50}$ of FeLV. mRNA expression of the indicated genes was measured by real-time qPCR and normalized to expression of two feline housekeeping genes (*GUSB* and *YWHAZ*) at time points indicated for each experiment. Depicted expression factors at each time point represent the ratio of measured mRNA levels in the infected samples compared to samples of non-infected cells. Only genes indicating at least a 2-fold modulation in their expression at any time point are shown. Data represent means of duplicate reactions for each time point. Standard deviations are shown as an indication for reproducibility.

879 **Figure 3.** Innate immune responses to FIV infection of feline PBMCs in vitro. PBMCs of
880 one cat were inoculated with 50 $TCID_{50}$ of FIV. mRNA expression of the indicated genes
881 was measured 3, 6, 12, 24 and 48 hours after inoculation and normalized to expression of
882 two feline housekeeping genes (*GUSB* and *YWHAZ*) at each time point. Depicted
883 expression factors at each time point represent the ratio of measured mRNA levels in the
884 IRM-stimulated samples compared to samples treated with a negative control. Only
885 genes indicating at least a 2-fold modulation in their expression at any time point are
886 shown. Data represent means of duplicate reactions for each time point. Standard
887 deviations are shown as an indication for reproducibility.
888
889 **Figure 4.** Measurement of *IFNα* expression levels in blood 1 week after FIV challenge
890 infection in vivo. mRNA expression of *IFNα* and two housekeeping genes (*GUSB* and
891 *YWHAZ*) from whole blood samples of 10 cats collected before and 1 week after
892 challenge infection with FIV was measured by real-time qPCR. DeltaCt values depicted
893 were calculated by substracting the average of 45-Ct values for two housekeeping genes
894 (*GUSB* and *YWHAZ*) from 45-Ct values for *IFNα* of the corresponding sample. *
895 p=0.0059

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Figure 1

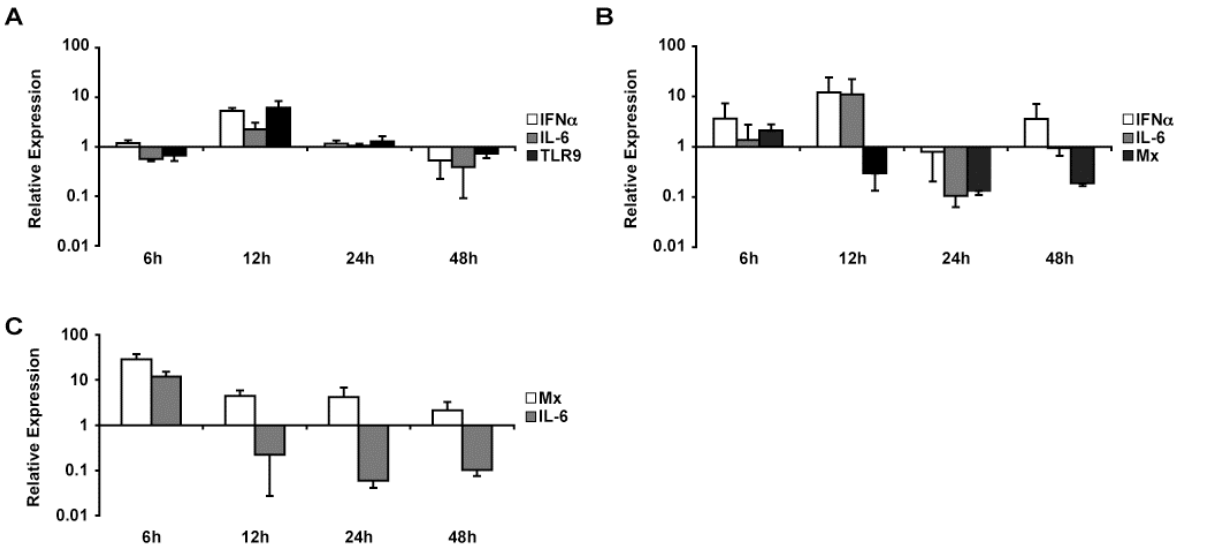


Figure 2

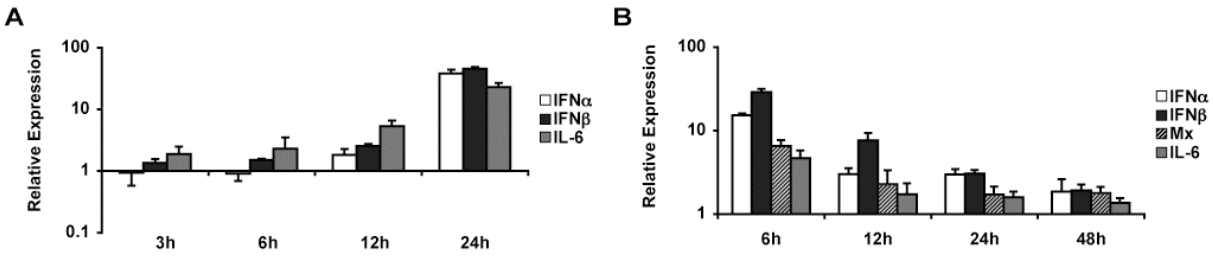


Figure 3

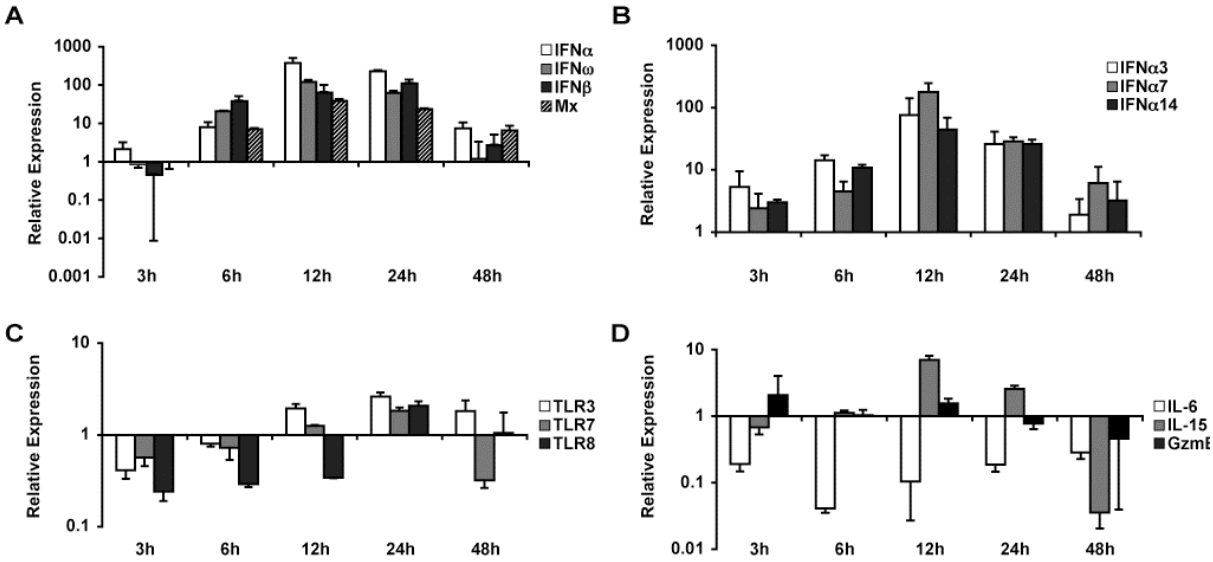
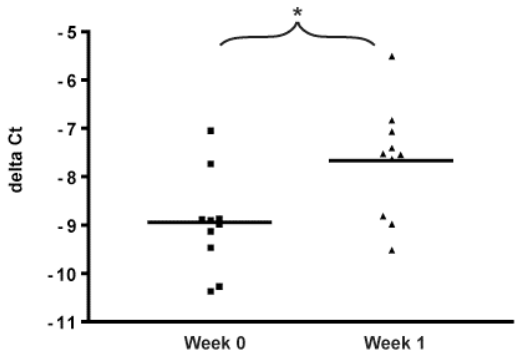


Figure 4



3.2 Manuscript 2

This second manuscript "*Stimulation with a Class A CpG Oligonucleotide Enhances Resistance of Feline Cells to Infection with Viruses from Five Different Families"* presents the results of an extensive analysis on the potential of a CpG-containing molecule to favourably manipulate the innate immune response of feline immune cells. The newly designed PCR assays introduced in the previous manuscript (section 3.1) are first utilized to depict the immunomodulatory effects of this IRM on the gene profiles of immune cells isolated from the blood of 14 individual cats from different age groups. The observations are supported by the measurement of other innate immune parameters on a protein level with methods such as flow cytometry and western blot. In a second phase, a series of cell culture experiments demonstrate the induction by this IRM of an "antiviral state" in target cell lines that subsequently display significantly enhanced resistance to 5 different feline viruses. Although the relevance of the findings of this study for the human population and the advantages of the feline model for further immunological studies are underlined in this article, the data is obviously also of primordial importance in veterinary medicine. The text was submitted recently to PLoS One and is currently under review.

Stimulation with a Class A CpG Oligonucleotide Enhances Resistance of Feline Cells to Infection with Viruses from Five Different Families

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Abstract

Emerging viral diseases represent a considerable threat to humans and animals worldwide and pressure the development of novel strategies to prevent pandemics. Oligonucleotides (ODN) containing cytosine-phosphate-guanosine motifs of class A (CpG-A) are highly potent synthetic inducers of innate antiviral mechanisms. The aim of

this study was to test their ability to prevent viral replication as stand-alone agents in an outbred species. We used the domestic cat as a model, as felids are naturally susceptible to infection by viruses resembling in their biological properties those affecting humans. CpG-A stimulation of feline peripheral blood mononuclear cells (PBMCs) enhanced their proliferation, increased the presence of co-stimulatory molecules major histocompatibility complex (MHC) II and B7.1 on their surface and influenced their gene expression profiles in an antiviral orientation. Notably, transcription levels of interferon (IFN)α, interleukin (IL)-6, IL-12, and IFNγ were increased by up to 12'000, 40, 25, and 12-fold respectively in PBMCs pulsed for 24 hours. Incubation of the supernatants of CpG-A stimulated PBMCs with feline cell lines of epithelial and fibroblastic origin induced expression of the antiviral myxovirus resistance (Mx) gene in these target cells, with a peak of up to 2000-fold increase in transcription after 6 hours and protein levels reaching a maximum within 24 hours and remaining stable for several days thereafter. Most importantly, the supernatant-treated cells showed significantly enhanced resistance to feline viruses from 5 distinct families, namely *Coronaviridae*, *Herpesviridae*, *Caliciviridae*, *Parvoviridae*, and *Retroviridae*. The rate of protection of the cells strongly correlated with the induction of type I IFN in the PBMCs and was concordant with the level of Mx expression in the target cells. Altogether, our data highlight the promising potential of CpG-A to induce a preventive antiviral state in outbred species and to protect populations against a broad range of virus infections.

Author Summary

New preventive strategies are required in order to limit the propagation of emergent viral diseases, such as severe acute respiratory syndrome (SARS) and avian influenza, which can endanger human and animal populations. In contrast to vaccination, protection conferred by modulation of the innate immune system holds the advantages of inducing immediate responses and overcoming barriers set by the inability to predict the exact nature of new pathogens. Synthetic oligonuleotides containing unmethylated cytosine-phosphate-guanosine motifs (CpG ODN) are readily recognized by mammalian innate immune cells and offer a powerful means to stimulate early defense mechanisms. For our studies related to innate immunity, we selected to work with the domestic cat, which belongs to an outbred species naturally affected by many viruses with human counterparts. Herein, we illustrate the broad immunomodulatory and antiviral properties of a class A CpG ODN (CpG-A) in feline cells. Stimulation of immune cells with this molecule increased their proliferation, promoted intercellular interactions, and induced production of soluble molecules that could impair replication of viruses from five families in feline target cells of various tissue origins. Our results underline for the first time the promising potential of CpG-A in generating general resistance to viral infection in an outbred species.

Introduction

Viruses represent a considerable threat to humans and animals worldwide. Although vaccination successfully confers protection to certain infectious agents, the currently most dangerous pathogens tend to be emerging and re-emerging viruses such as the severe acute respiratory syndrome (SARS)-associated Coronavirus, influenzaviruses, the

human immunodeficiency virus (HIV) and West Nile and Ebolaviruses, as they are often of unknown origin, insufficiently characterized or rapidly changing, and threaten to disseminate before a vaccine or appropriate treatment can be made available to the population. Thus, in order to diminish risks of pandemics, the development of additional prophylactic strategies is currently needed.

A promising addition to vaccination is the manipulation of innate immunity. Innate pathogen recognition relies on a set of sensory molecules, the Toll-like receptors (TLRs), which enable the immediate reaction of specific immune cells to pathogen "danger signals", the so-called pathogen-associated molecular patterns (PAMPs) [1]. Due to their abundance in all bacterial as well as some viral genomes, oligodeoxynucleotides (ODN) containing unmethylated cytosine–phosphate–guanosine (CpG) motifs are effectively recognized as PAMPs by the vertebrate innate immune system [2]. Response to CpG ODN stimulation is conferred through TLR9, expressed mainly in the intracellular compartments of human B cells and plasmacytoid dendritic cells (pDCs) [3,4]. Alarmed TLR9 is the initial instigator of gene expression profiles that strongly support antiviral mechanisms: upregulation of costimulatory molecules major histocompatibility complex (MHC) II, B7.1 and B7.2 on the surface of stimulated cells provides them with a stronger antigen presenting potential [5,6] and production of cytokines such as type I interferon (IFN), interleukin (IL)-12, IFNγ, IL-6 and tumor necrosis factor (TNF)α, contribute to providing an optimal immune environment for the development of innate and adaptive responses against intracellular pathogens [7,8]. Probably the most important antiviral property of CpG ODN resides in their potential to stimulate the production of high amounts of type I IFN by pDCs [9]. This family of cytokines, which includes IFNα,

IFNω and IFNβ, has been shown to considerably enhance natural killer (NK) cell cytotoxicity [10], promote differentiation, maturation and immunostimulatory functions of monocytes and DCs [11], induce B cell production of immunoglobulin [12] and T-helper (Th)1 differentiation of T cells [13,14]. Moreover, upon binding to their ubiquitously distributed receptor, type I IFNs effectively induce the synthesis of various intracellular proteins which interfere with the replication of a broad range of viruses [15]. The myxovirus-resistance protein (Mx) GTPase is a well-studied example of these intracellular antiviral effectors. This enzyme is known to be directly regulated by the type I IFN, and its detection is readily used as marker for upregulation and biological activity of this cytokine family [16].

Distinct classes of ODN have been shown to induce differential responses by human cells [17]. Class A CpG ODN (CpG-A) consist of CpG motifs in a phosphodiester core, flanked on both ends by phosphorothioate poly (G) sequences. CpG ODN of this class are characterized by their potential to both induce massive type I IFN secretion by pDCs [9] and increase NK cytotoxicity [18], rendering them ideal candidates as prophylactic enhancers of innate immunity to viral infections. Conversely, class B CpG ODN (CpG-B), which encode multiple CpG motifs on a phosphorothioate backbone, promote monocyte maturation and B cell activation, thus substantially supporting the development of humoral immune responses [19,20,21]. Both CpG-A and CpG-B have indicated immunostimulatory properties in immune cells of mice, primates and many domestic species *in vitro* [22,23,24,25,26,27,28,29], while *in vivo* studies in outbred animals have mainly been carried out with CpG-B [21,30,31]. In an effort to combine the advantageous effects of both CpG-A and CpG-B, class C [32,33] and class P ODN [34] were developed in more recent years. Although *in vivo* use of these newer classes of CpG

ODN seems promising [35,36,37,38], CpG-A remain the strongest inducers of type I IFN described to date.

In light of their broad immunomodulatory properties, CpG ODN have made their way to human clinical trials as vaccine adjuvants [39] and as combinatorial or monotherapies for cancer [40], allergies [41] and chronic infectious diseases [42]. Meanwhile, their potential as prophylactic stand-alone inducers of innate defense mechanisms has been the subject of fewer studies. Most works in this field initially described protection of mice against bacterial [43,44,45,46,47] and parasitic [48,49,50] infections. More recently, induction of resistance to viral infections was shown also in mouse models for Herpes Simplex Virus [51], Vaccinia virus [52], neurotropic arenavirus [53] and foot and mouth disease virus [54]. With exception of the latter, all these studies were carried out with CpG-B. In an outbred species, partial antiviral protection has only been described in two studies so far, in which reduced shedding of herpes and parainfluenza viruses was observed in lambs after administration also of a CpG-B [55,56]. To our knowledge, prophylactic antiviral potential of CpG-A has not yet been described in outbred animals.

The feline model holds several advantages in studies related to innate immunity and viral diseases. First, in contrast to laboratory mice, cats belong to an outbred species, enabling more realistic consideration of individual variability in immunologic responses. Studies with felids also enable to circumvent issues related to the notable differences between the innate immune systems of mice and humans, including altered expression and repartition of TLRs [57,58], different NK cell activating ligands [57], distinctive stimulatory CpG ODN sequences [22] and absence of important antiviral mechanisms in inbred strains

[16]. Furthermore, while felids are naturally affected by viruses with biological properties very similar to those affecting humans [59], the necessity in murine models to either modify the infectious agent or to create transgenic animals considerably limit the possibilities to extrapolate data from mice to humans [57]. Finally, while feline viruses have acquired challenging propagation strategies throughout evolution, cats have retaliated with the development of a stark innate antiviral immune system. Altogether, almost 30 subtypes of feline type I IFN have been identified [60,61,62,63] and their biological properties characterized [63,64,65].

With the objective to characterize both the immunomodulatory and antiviral properties of CpG-A, we carried out a series of *in vitro* experiments and observed the induction of broad immunoprotective effects in the feline model. In addition to strengthening the antiviral qualities of feline immune cells, we found that the prototype of CpG-A, ODN 2216 [9], can stimulate the production by these cells of soluble molecules possessing inhibitory properties on the replication of five different families of viruses: *Coronaviridae*, *Herpesviridae*, *Caliciviridae*, *Parvoviridae*, and *Retroviridae*.

Results

Prototype CpG-A, ODN 2216, induce proliferation of primary feline immune cells and enhance their expression of costimulatory surface molecules

Proliferation of peripheral blood mononuclear cells (PBMCs) in response to treatment with CpG ODN gives strong indications about the biological activity of the stimulatory molecule and has been used to screen ODN in many species [22]. In relation to this, the potential of CpG-A to induce proliferation of feline PBMCs was assessed by

measurement of ^{3}H-thymidine incorporation after stimulation. Purified PBMCs from eight adult cats proliferated significantly in presence of ODN 2216 when compared to cells treated with either ODN 2243 (p= 0.0039) or phosphate buffered saline (PBS) (p=0.0078) as inactive controls (Fig 1A). The cells of four cats (c06, c08, c09, c12) could be stimulated to particularly high proliferative rates by ODN 2216 (values above the mean of eight cats illustrated in Fig1A) in the following order: c08>c12>c09>c06.

Another characteristic feature of stimulatory ODN is their ability to enhance interactions between various immune cell populations by upregulation of cell surface costimulatory molecules. With the objective to test whether ODN 2216 could exert such properties in feline immune cells, the expression of B7.1 and MHCII was measured by flow cytometry in stimulated PBMCs of the same eight cats as above. Difficulties linked to both limited availability of labeled antibodies for the feline species and few possibilities for co-staining of several markers and cell populations had to be circumvented in these experiments by evaluating the expression of both co-stimulatory molecules in gates defined to contain a PBMC population, a lymphocyte population and a non-lymphocyte population of cells. The observed effects varied considerably between the cells of individual cats, ranging from no alterations to an increase of 400% stained cells in some cellular subpopulations after ODN 2216 stimulation. Also, in some animals, stimulation with the control ODN 2243 indicated similar staining patterns as PBS, whereas in other cats the induction of effects comparable to those of ODN 2216 could be observed. The response of cells originating from a particular cat to stimulation with ODN 2243 did not however correlate with the influence of ODN 2216 on the expression of either surface molecule on these same cells. Altogether, a significantly larger percentage of cells within each gated population expressed higher B7.1 levels upon a 24-hour stimulation with

ODN 2216, when compared to stimulation with ODN 2243 or PBS (Fig 1B). Feline MHCII expression was assessed by differences in the mean fluorescence intensities in gated populations of differentially stimulated cells. Such measurements were selected because, in contrast to mice, MHCII is constitutively expressed in most cellular subpopulations of feline PBMCs. Overall, an increased expression of MHCII was measured in gated PBMCs as well as in cells gated as lymphocytes after stimulation with ODN 2216 for 24 hours. In contrast, the expression of this surface molecule in cells gated as non-lymphocytes did not alter significantly (Fig 1C). The cells of the three cats (c08, c09 and c12) that exhibited the highest proliferation rates in response to ODN 2216 (Fig 1A) also indicated the strongest increase in expression of both cell surface molecules in all cellular subpopulations tested.

ODN 2216 influence type I IFN and proinflammatory gene expression in primary feline immune cells

Through interaction with the TLR9, CpG-A typically induce expression of both type I IFN and proinflammatory cytokines in stimulated cells [9]. In order to understand whether ODN 2216 exert similar effects in the cat, treated cultures of feline PBMCs, FEA, CrFK and fcwf-4 cells were systematically screened for increased mRNA expression of IFNα and IL-6 following stimulation. All tested immortalized feline cell lines failed to respond to stimulation with ODN 2216 (Fig 2A and data not shown). However, this molecule exhibited potent immunomodulatory properties in feline PBMCs: when measured 24 hours post stimulation, a concentration of only 1µg/ml ODN 2216 was sufficient to enhance transcription of IFNα by 40-fold, and a maximum induction of this gene was observed when 4µg/ml ODN were used (Fig 2B). Although

affected in a similar pattern, the mRNA expression of the proinflammatory cytokine IL-6 remained comparatively low at all concentrations tested (Fig 2B). In experiments foreseen to determine gene expression kinetics in feline cells after stimulation with the ODN 2216 molecule, an influence on IFNα transcription could be measured as early as 3 hours after treatment of PBMCs, whereas increased levels of IL-6 mRNA were observed only as of 6 hours post stimulation. Notably, the highest induction of both genes was measured 24 hours after addition of ODN 2216 to the cultures, with transcription increasing by 9000-fold and 39-fold at this time point for IFNα and IL-6 respectively (Fig 2C). The observed effects were specifically conferred by the CpG motifs comprised in the 2216 ODN, since specific control ODN 2243 only induced slight elevations in the expression of the genes tested (Fig 2B and 2C). Importantly, trypan blue exclusion experiments indicated no evidence of cellular toxicity after treatment neither with ODN 2216 nor with ODN 2243 at all concentrations tested (data not shown).

ODN 2216 broadly influence the gene expression profile in primary feline immune cells of adult cats

In an effort to assess both the breadth of the effects conferred by treatment with a CpG-A and possible variability in the responses obtained in individual cats, the mRNA expression of 10 genes relevant to early immune responses was measured in ODN 2216 stimulated PBMCs of fourteen cats divided in four different age groups (group 1: 10 weeks (n=4), group 2: 1.5 years (n=4), group 3: 7 years (n=4), group 4: 14 years (n=2)). Only slight individual variability in gene induction was observed after 24 hours stimulation of immune cells from adult cats ranging between 1.5 and 14 years of age (Fig 3A, groups 2-4). Overall, the mRNA expression profile measured in stimulated PBMCs

of these cats corroborated their induction of strong antiviral immune responses. The induction of type I IFN mRNA, including IFNα, IFNβ and IFNω was substantially higher in the immune cells from every adult cat, with minimal inductions of 490, 60 and 1600-fold respectively observed in the older animals of group 4. Moreover, all individual IFNα subtypes tested were induced at similar levels in the cells of four adult cats from group 2 (Fig 3B). Increased levels of proinflammatory cytokine mRNA were also measured in most individuals of groups 2-4, with IL-6 more systematically increased than TNFα. The cells from these cats indicated a typical Th1 orientation after stimulation, with enhanced transcription of IL-12 in 9/10 and IFNγ in 6/10 animals, together with absent or only low induction of IL-4. Stimulation of PBMCs with ODN 2216 also created an optimal environment for NK cell activity, as indicated by the increases in mRNA expression of NK cell stimulator IL-15 by up to 20-fold and NK cell effector Granzyme B by up to 7-fold. The cells of those cats (c06, c08, c09, c12) that had most effectively proliferated (Fig 1A) and/or exhibited the strongest expression of co-stimulatory molecules (Fig 1B and 1C) following ODN 2216 stimulation also consistently expressed the highest mRNA levels of IFNα, IFNω, IL-6, Il-12, IL-15 and Granzyme B. PBMCs of cat c08 (group 2) were thereby by far most responsive to stimulation with ODN 2216. When cells from kittens of group 1 were stimulated, higher individual variability was observed. The PBMCs of only 2 out of 4 cats from this group could be stimulated with ODN 2216 to increase mRNA expression of the tested genes. In both individuals, not only the mRNA expression of type I IFN genes was enhanced at much lower levels than observed in adult cats but the overall immune response favored a Th2 direction, characterized by upregulated IL-4 and downregulated IL-12 mRNA levels (Fig 3A, group 1).

In order to determine whether a discrepancy in expression of TLR9 between the PBMCs of adult animals and kittens could play a role in these observations, mRNA levels of this gene were measured in immune cells of each cat. Although basal TLR9 expression was similar in the PBMCs of the cats of all age groups (Fig 3C, top panel), ODN 2216 stimulation increased TLR9 transcription in the cells of kittens, but decreased transcription of this gene in the cells of adult cats so that differences in the mRNA levels of this receptor were significantly higher in young cats (group 1) than in adult cats of groups 2 and 3 ($p=0.0286$) (Fig 3C, bottom panel).

ODN 2216 induce the production of soluble molecules that activate intracellular antiviral mechanisms in feline target cells

Protective properties of type I IFN against viruses originate from their ability to trigger the production of potent antiviral proteins in nearby cells. The expression of one of these antiviral proteins, the Mx GTPase, is known to be directly stimulated by type I IFN and can be used as marker for the induction of intracellular antiviral mechanisms by these cytokines in cats as well as in other species [16,66]. Thus, as detection of feline type I IFN on a protein level is rendered difficult by the lack of antibodies on the market specifically recognizing these proteins, mRNA levels of Mx were measured in feline PBMCs after stimulation with ODN 2216 as indication for production of type I IFN. Transcription of Mx was significantly enhanced and remained proportional to mRNA expression of type I IFN in stimulated PBMCs of individual cats (Fig 4A). Moreover, peak levels of Mx mRNA were measured 24 hours after stimulation of the cells, indicating the presence of optimal effects of the type I IFN present in the cell culture medium at this time point (Fig 4B). In order to further determine the potential of the type

I IFN liberated by PBMCs after ODN 2216 stimulation to induce intracellular antiviral mechanisms in non-immune target cells, cell-free supernatants of PBMCs derived from the blood of individual adult cats and stimulated *in vitro* for 24 hours with ODN 2216, ODN 2243 or endotoxin-free PBS (Sup 2216, Sup 2243 and Sup Neg respectively), were incubated with CrFK and fcwf-4 cells. Mx gene transcription was significantly enhanced in cells treated with Sup 2216 compared to Sup 2243 ($p=0.0078$) and Sup Neg ($p=0.0078$), at levels comparable to those achieved by stimulation of the cells with 100U recombinant feline IFNα (rfeIFNα) (Fig 4C and data not shown). The Sup 2216 derived from cells of individual animals (c06, c08, c09, c12), that had shown particularly strong responses to ODN 2216 in previous experiments, also induced the highest Mx mRNA expression in both cell lines tested. Although treatment with Sup 2243 systematically increased Mx transcription in target cells, protein levels remained low (Fig 4C and data not shown). Furthermore, peak induction of Mx transcription was observed in both cell lines already within 6 hours of incubation with Sup 2216, while protein levels achieved maximum levels within 24 hours post stimulation and remained stable thereafter at least for another 24-48 hours (Fig 4D and data not shown).

ODN 2216 inhibit replication of common feline viruses in vitro

Felids are frequently affected by four viruses of different families: the feline herpes virus (FHV), calicivirus (FCV), parvovirus (FPV) and coronavirus (FCoV). Although these viruses cannot productively infect purified PBMCs *in vitro*, they share the ability to induce cytopathic effects (CPE) in CrFK and fcwf-4 cells. These feline cell lines, however, do not alter their mRNA levels of genes selected as markers for innate immunity upon direct treatment with ODN 2216 (Fig 2A and data not shown), preventing

from assessing the potential of this molecule to inhibit viral replication directly in these cells. Thus, CrFK and fcwf-4 cells were incubated, prior to their inoculation, with the cell-free supernatants of PBMCs mentioned above: Sup 2216, Sup 2243 and Sup Neg. To this aim, supernatants derived from PBMCs treated for 24 hours were selected, as Sup 2216 produced by the cells of several adult cats were estimated to contain optimal type I IFN amounts at this time point, according to the induction of Mx transcription measured directly in these immune cells (Fig. 4A and 4B). CrFK and fcwf-4 target cells were then incubated with the supernatants for 24 hours before inoculation, as this time span had indicated highest induction of antiviral mechanisms (Fig 4D). The antiviral effects of the supernatants were initially tested on vesicular stomatitis virus (VSV) as control, as this virus is widely recognized for both its potential to induce CPE in cell lines of multiple species and for its particularly high sensitivity to the effects of type I IFN [67]. When the Sup 2216 derived from the PBMCs of eight adult cats that had broadly responded to *in vitro* ODN 2216 stimulation (Fig 3A, groups 2 and 3) were incubated with fcwf-4 cells prior to their inoculation, significant inhibition of VSV replication was observed ($p=0.0039$) (Fig 5A). The replication of this virus was also to some degree repressed by Sup 2243 ($p=0.0078$), an observation reminiscent of the slight induction of Mx in target cells incubated with these supernatants (Fig 4C). In turn, the propagation of FCV, FCoV, FHV and FPV on fcwf-4 cells was also significantly suppressed by the Sup 2216 when compared to Sup 2243 ($p=0.0039$, $p=0.0039$, $p=0.0078$ and $p=0.0039$ respectively) and Sup Neg ($p=0.0039$), however with expected lower sensitivity than VSV (Fig 5A and Table 1). Both Sup 2243 and Sup Neg failed to inhibit replication of this heterogeneous group of feline viruses, underlining the essential role of the 2216 molecule in conferring the observed effects. Importantly, cells stimulated with ODN 2216 directly did not

indicate any resistance to viral replication, in concordance to their impaired response to this molecule already measured on a genetic level (Fig2A and data not shown). With respect to the younger cats, the Sup 2216 of those 2 kittens whose cells responded to ODN 2216 stimulation (Fig 3A, group 1) could also inhibit both VSV and FCV on fcwf-4 cells, while the supernatants derived from the PBMCs of the other 2 kittens indicated no inhibition potential on these viruses (Fig 5C). Altogether, the suppression of viral replication by the Sup 2216 of the cats could be compared to that conferred by treatment of the cells with 10 to 100U rfeIFNα (data not shown), and induction of Mx target cells by the supernatants highly correlated with the inhibition of all viruses (Fig 5B). Finally, Sup 2216 derived from PBMCs of cats c06 and c08, whose cells had indicated strong responsiveness to stimulation with ODN 2216 in previous experiments, most efficiently inhibited the replication of all viruses.

Similar results were notably obtained when the supernatants of PBMCs derived from all cats were incubated with CrFK cells prior to their inoculation with all the above-mentioned viruses (data not shown). This cell line has previously indicated less sensitivity to the antiviral effects of type I IFN [68] and concordantly, average fold viral inhibition in CrFK cells in our experiments was approximately half that observed in fcwf-4 cells.

ODN 2216 inhibits replication of a retrovirus in vitro

The life cycle of retroviruses is characterized by the reverse transcription of their genomic RNA into DNA and subsequent integration of this viral DNA as provirus into the genome of the host, causing permanent infection most often accompanied by persistent virus production by infected cells. The feline leukemia virus (FeLV), a

gammaretrovirus that can be propagated on FEA cells *in vitro*, affects domestic cats worldwide. As viral replication in chronically infected cats can be lowered by treatment with IFNα [69], the potential of Sup 2216 produced by the PBMCs of five adult cats (c06 and c08 from group 2; c09, c10, c12 from group 3) to inhibit productive infection of FEA cells was analyzed. In initial experiments, this cell line exhibited similar responses as fcwf-4 and CrFK cells to both direct treatment with ODN 2216 and incubation with the different supernatants (Fig 2A, 4C and data not shown). Compared to incubation with medium alone, treatment of FEA cells with Sup 2216 for 24 hours prior to inoculation with FeLV followed by repetitive treatments of the cells with this supernatant every 2 days thereafter significantly reduced viral RNA ($p<0.05$) and DNA ($p<0.05$) measured in the cell culture supernatants and cells respectively as of 4 days post inoculation (Fig 6A). The antiviral potential of the supernatants from the individual cats was very similar; however the best results were conferred by the Sup 2216 derived from cells of c08 (depicted in Fig 6A). The kinetic curve of viral RNA loads in cultures of cells treated with Sup 2216 of this cat was similar to those obtained in cells treated with 50U rfeIFNα (data not shown). Also, treatment of the cells with ODN 2216 directly affected neither viral RNA nor viral DNA loads measured in the supernatants and the cells respectively (data not shown). Mx mRNA expression was 80-fold higher in the Sup 2216 treated cells than in all controls 8 days post inoculation, indicating the ability of these supernatants to sustain antiviral mechanisms when applied to the cells repeatedly (Fig 6B). Furthermore at this time point, the Sup 2216 treated cells exhibited significantly lower viral DNA loads ($p=0.0313$) and produced significantly less virus ($p=0.0313$) than cells treated with Sup 2243, Sup Neg or medium alone (Fig 6B). The extent of Mx transcription conferred by the Sup 2216 of the cats strongly correlated with lower provirus ($p=0.0053$) and virus

(p=0.0012) loads measured in the FEA cells and supernatants respectively on day 8 post inoculation (p=0.0053). Also, the highest Mx mRNA levels in target cells were conferred by Sup 2216 of cat c08 and reflected by the lowest viral and proviral loads measured at this time point in our experiments.

Discussion

In the present study, we show that the broad immunomodulatory effects of CpG-A in feline cells could prophylactically inhibit the replication of five feline viruses from different families, namely FCV, FPV, FHV, FCoV and FeLV. ODN 2216, the first described CpG-A [9], could stimulate feline PBMCs to produce soluble molecules that significantly increased resistance of various feline target cell lines to viral propagation. The observed repression of viral replication highly correlated with the mRNA expression of type I IFN genes in stimulated PBMCs as well as the induction, prior to inoculation, of antiviral mechanisms in the target cells. To our knowledge, this is the first study reporting efficient inhibition of viral replication by a CpG-A in an outbred species. Furthermore, our results demonstrate the potent prophylactic potential of a synthetic molecule as stand-alone agent against a large range of viral pathogens simultaneously.

Cells of the feline immune system were strongly influenced when cultured in the presence of ODN 2216. The CpG motifs comprised in the molecule were essential contributors to the immunomodulatory and antiviral effects of this molecule, while the sequence of flanking nucleotides, the single-stranded structure as well as the synthetic nature of the backbone thereby played a minor role, as indicated by the only slight stimulatory potential of the GpC control ODN 2243 in most experiments. As shown in

this study in experiments conducted with PBMCs of eight to fourteen SPF cats, ODN 2216 significantly altered the behavior and phenotype of feline immune cells.

First, feline PBMCs significantly proliferated in presence of ODN 2216 (Fig 1A), indicating a direct and /or indirect stimulation of one or more immune cell subpopulations by this molecule. Although in contrast to other classes of ODN, CpG-A does not generally induce lymphocyte proliferation in mice [9], similar effects have previously been observed in an outbred species, where higher concentrations of CpG-A could induce minimal levels of proliferation in ovine cells [70].

Additionally, ODN 2216 increased the expression of cell surface co-stimulatory molecules in PBMCs (Fig 1B and 1C), reinforcing possible interactions between various immune cell populations. While B7.1 molecules were upregulated on both lymphocytic and non-lymphocytic cells after ODN 2216 stimulation, MHCII expression could only be increased on lymphocytic cells. This observation may be linked to the necessity of transport to the cell surface of MHCII molecules coupled with antigen for presentation to other immune cells. Also, not only the presence of CpG motifs but also the chemical structure of the ODN seemed to play an important role in the upregulation of MHCII and B7.1 co-stimulatory molecules in feline PBMCs, as control ODN 2243 also significantly increased expression of both B7.1 and MHCII in stimulated lymphocytic cells. The potential of synthetic backbones to enhance cell surface molecule expression in immune cells has indeed already been reported [71]. It should also be noted that results in these experiments indicated relatively high variability among the cats, reflecting the heterogeneity of PBMC subpopulations in individuals of the same species. Differential gating adapted to the PBMC phenotypes of each animal or differentiation of various

immune cell subpopulations by co-staining for appropriate markers could confer further information concerning intraspecies stimulatory variability.

Finally, the transcription of a series of markers of innate immune responses was considerably influenced in feline PBMCs by stimulation with ODN 2216. The most astonishing effects of this molecule remain the potent induction of IFNα and IFNω, with mRNA expression of these genes increased by up to 12'000 and 35'000-fold respectively in PBMCs of certain animals (Fig 3A). In line with observations published recently, the induction of IFNγ by this CpG ODN remained moderate [72]; nevertheless, the measurements carried out 24 hours after stimulation of purified feline cells support induction of Th1-oriented immune responses and enhanced NK cell activity, both highly desired in the contexts of viral infection.

Altogether, although the immune cells collected from cats of 14 years of age seem to respond slightly less well to ODN 2216 than those of younger adult animals (Fig 3A), consistency in stimulatory potential of this molecule among adult animals was reflected through the highly similar patterns in gene expression profiles induced in immune cells after stimulation as well as the moderate deviations in those experiments aiming the characterization of immunomodulatory properties of ODN 2216 (Fig 1A, 1B, 1C and Fig 3A, groups 2-4). The cells of several animals however, in particular cat c08, but also c06, c09 and c12, indicated particularly strong responsiveness to stimulation throughout the study. Such observations are most likely linked to the genetic variability of individuals of an outbred species, as inherited factors are known to play an important role in the magnitude of innate immune responses [73]. With respect to this observation, it should be noted that the SPF origin and maintenance in a barrier facility of the animals included in this study may slightly lower the variability in strength and breadth of innate immune

responses, and studies with cells obtained from field cats would give further information on divergence in response to stimulation of the innate immune cells of individual animals.

Notably, the immune cells of kittens indicated much more reticence to stimulation, with either limited or absent upregulation of both type I IFN and other genes measured after incubation with ODN 2216. Stimulated PBMCs from this group of young animals moreover indicated a tendency to develop an immunologic environment with a Th2 orientation, including higher production of IL-4 and impaired induction of IL-12 compared to cells from adult cats. Although the kittens included in this study were already of 10 weeks of age, these observations strongly corroborate the immature IFN system of neonatal mice [74], the impaired immune cell activation via TLR9 in human neonatal mononuclear cells [75,76] and the bias towards Th2 rather than Th1 responses in human fetuses and neonates [77,78].

Furthermore, concordantly to findings in human neonatal blood [79,80], basal TLR9 transcription in kittens indicated levels similar to those of adults. However, ODN 2216 stimulation increased mRNA expression of this gene only in the young animals (Fig. 3C). The lack of specific antibodies to feline TLR9 unfortunately renders further investigations on the mechanism behind these results and their implications in CpG ODN stimulation in kittens rather challenging. This observation may however be linked to the accumulation of TLR9 proteins in the endoplasmic reticulum of adult immune cells as a pool awaiting transport to the endosomes upon ligand-induced stimulation. Such a reserve of TLR9 proteins could substantially decrease the necessity of upregulation on the mRNA level [81]. Young individuals hypothetically have yet to establish this TLR9 pool in the endoplasmatic reticulum, leading to increased transcription of this gene

following stimulation with CpG ODN. Altogether, the results obtained from this extensive study regarding the effects of CpG-A on feline immune cells further support the suitability of the feline model for studies concerning early immune mechanisms and open new perspectives on possible explanations for the qualitative discrepancy between innate immune responses in newborns and adults.

Preventive treatment of cells with CpG ODN strengthens considerably the antiviral defense mechanisms of these and surrounding cells, restricting viral infections, and thus tilting the immunological balance in favor of the host. Concordantly, in infections with rapidly dividing pathogens, optimal protection of mouse models can be observed when CpG ODN are administered prior to infection [82]. Our data demonstrate that five viral species belonging to the Calicivirus, Herpes virus, Parvovirus, Coronavirus and Retrovirus families were sensitive to the immunologic effects of ODN 2216 in cats. Interestingly, although a recombinant feline type I IFN marketed in both Japan (Intercat®) and Europe (Virbagen Omega®) has made its way into therapeutic protocols for FCV, FHV, FeLV and canine parvovirus infections [69,83,84,85,86,87,88] and has demonstrated preventive capacities in a cattery developing an outbreak of FPV [89], CpG ODN have not yet been tested in any clinical settings in this species. In view of the potent induction of IFNα by CpG-A, it seemed reasonable to anticipate viral inhibition potential by this class of ODN. Surprisingly, CpG-A have shown prophylactic potential only in a mouse model of orthopoxvirus infection [52], and no studies have been conducted to date with this class of ODN in outbred species, even though strong immunomodulatory properties of this molecule have already been reported in cows, sheep, pigs and primates *in vitro* [26,28,29]. Moreover, when compared to direct

initiation of antiviral mechanisms by a recombinant IFNα protein, administration of CpG ODN holds the advantage of inducing the production of all type I IFN and their subtypes (Fig 3B), which have been shown to possess differential antiviral properties [90]. Also, CpG ODN specifically target dendritic cells (DCs), a non-lymphocytic cell population widely recognized to link innate and adaptive immune mechanisms. Modifications in the gene expression profile of non-lymphocytic cells by ODN 2216 enhances the expression of costimulatory molecules on their surface (Fig 1B) as well as the secretion of a broad range of cytokines (Fig 3A), both mechanisms enabling further transfer of the stimulatory signals to cells of the innate and specific immune systems.

In view of future *in vivo* experiments, a 3-step *in vitro* system was conceptualized and utilized in this study: purified PBMCs were first stimulated with ODN 2216 to secrete soluble molecules, including type I IFN, into the culture medium; cell-free supernatants of the stimulated PBMCs were then incubated with a target cell line reminiscent of epithelial (CrFK) or fibroblastic (fcwf-4, FEA) tissue; target cells were finally inoculated with a feline virus and viral replication was monitored. This method seemed much more suitable than direct stimulation of CrFK, fcwf-4 and FEA cells, as it enabled to circumvent the irresponsiveness of these immortalized cell lines to stimulation with ODN 2216 (Fig 2A), which is most probably linked to their insufficient differentiation for expression of the measured cytokines. Additionally, this system closely mimics an *in vivo* situation, as PBMCs comprise pDCs, the main players involved in recognition and response to CpG-A, enable interplay between heterogeneous populations of immune cells and circulate systemically, affecting many different tissues with the soluble molecules they produce. This method had already been utilized successfully in a study screening

525 CpG ODN for prevention of SARS [91]. Furthermore, the variety of soluble molecules
526 present in the supernatants of CpG-treated cells were found to be responsible for the *in*
527 *vivo* effects observed in a trout model [92], supporting the feasibility of this method as
528 primary model prior to *in vivo* studies.
529
530 The supernatants of PBMCs stimulated with CpG-A contain a mixture of molecules that
531 could play a role in the total antiviral effects observed. However, the viral inhibition was
532 both similar to that obtained after treatment of the cells with rfeIFNα and highly
533 correlated with the induction of Mx transcription in the cell lines incubated with the
534 supernatants (Fig 5B), underlining the central role of type I IFN in protection of target
535 cells against viral invasion. Inhibition of viral replication in the present study moreover
536 corroborates the sensitivity of the individual viruses to various subtypes of recombinant
537 feline interferon reported in past studies [65,68]. Importantly, Mx mRNA and protein
538 could not be directly linked to inhibition of the viruses tested, but rather used as markers
539 for induction of an antiviral state in tissue cells. Although the expression of other
540 antiviral molecules such as 2'5'oligoadenylate synthetase (OAS) and the RNA-dependent
541 protein kinase (PKR) was not measured, their induction has been reported following CpG
542 ODN stimulation of PBMCs in other species [93,94] and differential interplay between
543 several effector mechanisms most likely leads to the inhibition of individual viruses.
544 After a single 24-hour treatment with supernatants of ODN 2216 treated feline PBMCs,
545 an increased amount of Mx protein could be detected for at least 72 hours in all target
546 cell lines tested (Fig 4D), corroborating the kinetics of this protein following IFNα
547 stimulation reported in various cell types previously [95,96,97]. Also, when fcwf-4 cells
548 were incubated with the supernatants for 72 instead of 24 hours, similar inhibition of

VSV and FCV was observed (data not shown), further supporting possible protection against viral infection by ODN 2216 for at least several days, a phenomenon described already in mouse models [82].

The characteristic structure of a CpG-A molecule includes poly (G) sequences on a phosphorothioate backbone at both ends and CG dinucleotides embedded in a central palindromic sequence with phosphodiester linkages [9]. The necessity for poly (G) motifs renders CpG-A more difficult to produce than other classes of CpG ODN [29]. However, when considering the *in vivo* use of stimulatory molecules, CpG-A hold several considerable advantages. First, they remain the most potent inducers of type I IFN, themselves the most biologically active antiviral molecules known to date [16]. Also, as a result of their poly (G) stretches, they can form G-tetrads, highly ordered structures with enhanced stability [9]. Their only partial phosphorothioate structure could moreover help reduce possible long-term side effects related to synthetic backbones [98,99]. Finally, the flu-like symptoms generally linked with administration of immunostimulatory molecules *in vivo* may remain minor in cats treated with ODN 2216, since we observed only marginal increase in mRNA expression of the proinflammatory cytokines IL-6 and TNFα in stimulated immune cells when compared to type I IFN genes (Fig 2B and 2C). Altogether, our data reflect strong potential for this molecule in the prevention of a large variety of viral diseases, underlining the importance of developing newly structured molecules or of modifying administration strategies in order to both profit from the stimulatory sequence of ODN 2216 and circumvent safety, stability and manufacturing issues. Future *in vivo* studies with such newly designed ODN

could substantially support the development of powerful and convenient immune response modifiers for the prevention of viral infections in humans and animals.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with regulations of the Swiss law for animal protection (SR 445.1). The Veterinary Office of the Swiss Canton of Zurich officially revised the protocol and approved the study (Permit no. TVB 99/2007 and TVB 100/2007). The animals were housed in groups in an animal-friendly barrier facility under optimal ethological conditions [100]. For blood collections, the cats were sedated with a combination of ketamin and midazolam, and all possible efforts were made to minimize stress and suffering.

Animals

Fourteen male castrated SPF cats divided in four different age groups were used in this study: group 1 (c01-c04, 10 weeks), group 2 (c05-c08, 1.5 years), group 3 (c09-c12, 7 years) and group 4 (c13 and c14, 14 years). Cats c13 and c14 from group 4 originated from the same litter; all other individuals were not related to each other. The animals were purchased from Liberty Research Inc. (Waverly, NY, USA) and their SPF status was verified as previously described [101]. Blood samples were collected from the jugular vein under sterile conditions with K3-EDTA VACUETTE® tubes (Greiner Bio-One, St.Gallen, Switzerland) and a BD Vacutainer® Luer Adapter (Becton Dickinson, Allschwil, Switzerland). PBMCs or PBMC supernatants utilized in the separate

experiments were obtained from those animals mentioned in figure captions or annotated in the figures.

Feline PBMC isolation, cell lines, cell culture and cell viability assay

Feline PBMCs were isolated from EDTA-anticoagulated whole blood by Ficoll-Hypaque density gradient centrifugation using a standard protocol [102]. Purified cells were counted as described previously [103], using the Sysmex XT 2000iV (Sysmex, Norderstedt, Germany), prior to their utilization in the different experiments, and cultured in RPMI 1640 with Glutamax I (Gibco®, Invitrogen, Basel, Switzerland).

Adherent Crandell-Rees feline kidney (CRFK) cells (ATCC no. CCL-94) and Feline Embryonic Fibroblasts (FEA) were maintained in RPMI 1640 with Glutamax I, while adherent Felis catus whole fetus-4 (fcwf-4) cells (ATCC no. CRL-2787) were cultured in EMEM (ATCC 30-2003). All media were supplemented with 10% heat-inactivated fetal calf serum (Bioconcept, Allschwil, Switzerland), 100 U/ml penicillin and 100mg/ml streptomycin (Gibco®, Invitrogen).

The CpG-A prototype, ODN 2216, the control ODN 2243 (Alexis biochemicals, Enzo Life Sciences AG, Switzerland) and recombinant feline IFNα (rfeIFNα) (PBL Biomedical, Piscataway, New Jersey, USA) were solubilized in endotoxin-free PBS. ODN 2243 consists of the same sequence as ODN 2216, with CpG motifs inversed to GpC. In all experiments, both ODNs and rfeIFNα were diluted in RPMI 1640 with Glutamax I supplemented as described above.

Viability of stimulated cells was compared using the trypan blue exclusion test. Briefly, cells were stimulated for 24 hours with increasing concentrations ranging from 1µg/ml to 16µg/ml ODN 2216, ODN 2243 or equivalent volumes of PBS as control. Cells were

then detached with 0.05% trypsin-EDTA (Gibco®, Invitrogen) and stained with a 0.4% trypan blue solution (Dr. Bender and Dr. Hobein AG, Zurich, Switzerland). Both viable unstained and non-viable stained cells were microscopically counted and percentages of viable cells were compared.

Proliferation assay

PBMCs were seeded immediately after isolation at a concentration of $3x10^6$ cells/ml in 96-well U-bottom plates. Triplicate cultures for each cat were treated with either 4µg/ml ODN 2216 or 2243 or an equal volume of endotoxin-free PBS. After an initial incubation of 18 hours, the cells were pulsed for 24 hours with ^{3}H-thymidine (Perkin Elmer, Schwerzenbach, Switzerland). Standard liquid scintillation protocols were used for harvesting of the cells and uptake of ^{3}H was assessed with the Packard Tri-Carb 1600TR liquid scintillation analyzer (Perkin Elmer). Proliferation rates were calculated as the mean counts per minute (c.p.m) of triplicate cultures.

Flow cytometry

PBMCs were treated at a density of $3x10^6$ cells/ml with 4µg/ml ODN 2216 or ODN 2243 or an equivalent volume of endotoxin-free PBS and cultured for 24 hours in a 12-well format. During collection of the cells, the adherent cell fraction was removed with 0.05% trypsin-EDTA (Gibco®, Invitrogen). Harvested cells were divided into 3 fractions labeled separately with either anti feline B7.1 mouse monoclonal IgG (kindly provided by Prof Mary Tompkins of the flow cytometry and cell sorting laboratory, NC State College of Veterinary Medicine, USA), anti feline MHCII mouse monoclonal IgG1 (Department of Pathology, Microbiology and Immunology, University of California,

Davis, USA) or fluoresceinisothiocyanate (FITC)-conjugated mouse IgG1 as isotype control (BD Bioscience, Allschwil, Swizerland). The fractions were subsequently stained with R-Phycoerythrin (RPE)-conjugated goat anti-mouse IgG1 (BioConcept, Allschwil, Swizerland). Fluorescence data was obtained using the FACSCalibur® instrument (Becton Dickinson) and the CellQuestPro™ software. Gates representing lymphocyte and non-lymphocyte populations were set on the basis of forward versus side scatter, and a total of 50'000 events were acquired in the non-lymphocyte gate. Data was analyzed with the FlowJo software (Tree Star, Olten, Switzerland), whereby an additional gate was set comprising both lymphocyte and non-lymphocyte populations (PBMC gate). MHCII expression was determined as geometric mean of fluorescence intensity for each gated cell population. In order to asses expression of B7.1 molecules, a cut-off for positive events was determined for each subpopulation of gated cells, where less than 2% of the negative events were included in the high analysis region. Identical gates were set for all cats in such a way that they comprise the desired cell populations of each individual.

Relative gene expression analysis

All experiments were carried out in a 96-well format. PBMCs were stimulated with ODN 2216, ODN 2243 or endotoxin-free PBS at a density of $3x10^6$ cells/ml directly after isolation, while CrFK, FEA or fcwf-4 cells were cultured to confluency prior to stimulation. For stimulation of adherent with supernatants (production see below), total cell culture medium was discarded from the wells and the monolayers were further cultured in 100µl undiluted supernatants for the rest of the experiment. At time points relevant to each experiment, the supernatants were removed and cells were lyzed with mRNA lyzis buffer (mRNA isolation kit I, Roche Diagnostics, Rotkreuz, Switzerland).

mRNA extractions were performed with the mRNA Isolation Kit I and MagNA Pure LC Instrument (Roche Diagnostics) and first strand cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rotkreuz, Switzerland). Real-time quantitative PCR (qPCR) reactions consisted of 5µl cDNA in a total volume of 25µl per reaction using the TaqMan® Fast Universal PCR Master Mix (Applied Biosystems). Thermocycling conditions included an initial denaturation of 20s at 95°C followed by 45 cycles of amplification by melting at 95°C for 3s and annealing at 60°C for 45s. Primers and probes for feline genes have been previously described [104]. mRNA expression factors of selected genes, which correspond to ratios of mRNA levels measured in ODN 2216 and ODN 2243 stimulated versus PBS stimulated cells, were calculated and normalized with GeNorm version 3.5 [105], using either both feline β-glucuronidase (GUSB) and tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ) (usually) or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) alone (when specified) as reference genes, under conditions validated for the feline species [106]. Generally depicted in the graphs are mean expression factors calculated from duplicate experiments carried out simultaneously. Where results of one cat are shown, experiments were conducted with cells of at least 3 individual animals and representative data is shown.

Production of supernatants

For each cat, PBMCs were resuspended in supplemented RPMI 1640 with Glutamax I (Gibco®, Invitrogen) at a concentration of 10^6 cells/ml in 6 well plates and stimulated immediately after isolation with 4µg/ml ODN 2216, 4µg/ml ODN 2243 or an equivalent volume of endotoxin-free PBS. After 24 hours incubation, supernatants were harvested by

centrifugation of the cultures twice at 2000xg for 10 minutes, aliquoted and stored at -20°C. Large supernatant quantities were produced with PBMCs purified after one blood collection, enabling the utilization of the same supernatants for virus inhibition experiments concerning VSV, FCV, FCoV, FHV and FPV. New supernatants batches were produced for use in FeLV inhibition assays. Supernatants derived from PBMCs stimulated with ODN 2216, ODN 2243 and endotoxin-free PBS are referred to as Sup2216, Sup 2243 and Sup Neg in the text and in the figures.

Western Blot

CrFK and fcwf-4 grown to confluency in 12-well plates were stimulated with 600µl of PBMC supernatants produced as explained above. At the time points indicated, cells were harvested and counted. 10^6 cells of either cell line were resuspended in 30µl sample buffer (0.5M Tris(hydroxymethyl)aminomethane, 5% SDS, 10% β-mercaptoethanol, 40% glycerol, and 0.05% bromphenol blue) and boiled at 95°C for 5 minutes. SDS-PAGE separation and submersed immunoblotting procedures were carried out as previously described [97]. The Spectra Multicolor Broad Range Protein Ladder (Fermentas GmbH, St. Leon-Rot, Germany) served as molecular weight standard marker for each blot. For protein visualization, membranes were first cut immediately below the 80kB marker band. The top and bottom membrane fractions were incubated with murine anti-human Mx MAb M143 (generously provided by Dr. J. Pavlovic, Institute for Virology, University of Zürich, Switzerland) and murine anti β-actin monoclonal antibody as a loading control (Sigma Aldrich GMbH, Buchs, Switzerland) respectively. Both fractions were subsequently incubated with a peroxidase-labelled goat anti-mouse

IgG (Jackson Immunoresearch, Newmarket, Suffolk, UK). Bands were digitalized using the Chemigenius 2 BioImaging System (Syngene, Cambridge, UK).

Viruses and viral inhibition assays

VSV Indiana strain (Institute of Virology, Vetsuisse Faculty, University of Zurich, Switzerland), FCoV Wellcome strain (a generous gift from Prof. A. Kipar, University of Liverpool, Great Britain), FPV (kindly provided by Prof. U. Truyen, University of Leipzig, Germany), FHV ZH5-04 strain and FCV F9 strain (kindly provided by Veterinaria AG, Zurich, Switzerland) were titrated on both CrFK and fcwf-4 cells. Viral stock dilutions inducing 95% cytopathic effect (CPE) after 24 hours (72 hours for FCoV and FPV) were selected for inhibition experiments in order to ensure proper measurement of inhibitory effects. Monolayers of CrFK and fcwf-4 cells in 96-well plates were incubated for 24 hours with the 100µl of the supernatants produced with PBMCs from cats of groups 1, 2 and 3. With the exception of assays carried out with FPV, viral inhibition experiments were conducted simultaneously and with supernatants thawed an equal number of times. The treated cells were then inoculated with virus (VSV, FCV, FHV, FCoV) or trypsinized with 0.05% trypsin-EDTA (Gibco®, Invitrogen) and allowed to settle in viral suspension (FPV), and plaque assays were carried out after 24 hours (72 hours for FCoV and FPV) according to the procedure described previously [107]. Briefly, supernatants were discarded and cell debris was removed from the wells by 3 cycles of washing with Hank's balanced salt solution (HBSS) (Gibco, Invitrogen) and shaking on an orbital shaker for 15 seconds. Remaining cells were fixed with 5% formalin and stained with a crystal violet solution. For spectrophotometric measurements, 100% methanol was added to the dried out wells and

absorbance was read at 595nm on a SpectraMax Plus 384 microtiter plate reader (Molecular Devices, Bucher Biotec AG, Basel, Switzerland). Viral inhibition rates were calculated with the following formula:

Mean optical density (OD) values of duplicate wells treated with Supernatant / Mean OD values of quadruplicate wells treated with medium alone

FeLV-A Glasgow-1 strain (a generous gift from Proff. M. Hosie and O. Jarret, University of Glasgow, Great Britain) was titrated on FEA cells, and the lowest stock dilution leading to productive infection of the cells after 48 hours was used for inhibition experiments. Experiments were carried out in 96-well plates and cells were treated with 100µl of supernatants or relevant controls immediately prior to inoculation. Every second day thereafter, 50µl culture medium was replaced by the same volume fresh supernatant. At appropriate time points, cells and supernatants were harvested and total nucleic acid was extracted from both the cells and supernatants using the MagNA Pure LC DNA Isolation Kit I and MagNA Pure LC Instrument (Roche Diagnostics). Viral replication in supernatants and proviral loads in cells were measured by real-time RT-PCR and real-time PCR respectively, with assays previously described [108]. The time course experiments were conducted with supernatants derived from PBMCs of three cats and the measurements on day 8 post inoculation were carried out with material derived from two additional cats. In order to facilitate interpretation of the figures illustrating measurements of viral RNA loads, 45 cycles -cycle threshold (Ct) values were calculated and means of duplicate wells are depicted.

Statistical analysis

All statistical analyzes were performed using GraphPad Prism for Windows, version 3.0 (GraphPad Software, San Diego California USA). Due to the limited number of cats integrated in the study, we refrained from using a parametric approach in the statistical tests. As such tests require a larger sample size than n=4, it was not possible to calculate p-values for the induction in expression of each gene shown in figure 3. Differences between treatment groups in proliferation of PBMCs and expression of co-stimulatory molecules on the surface of these cells were analyzed with a Wilcoxon signed rank test, where values for each cat were paired. Relative Mx mRNA expression, OD values from plaque assays, ratios for FeLV provirus and Ct values for FeLV viral RNA loads were analyzed with a Wilcoxon signed rank test with pairing of values for each cat when treatment with different supernatants were compared, or a Mann-Whitney test when incubation with supernatant was opposed to treatment with medium alone or rfeIFNα. Normalized and relative TLR9 mRNA expression ratios between different animal age groups were also compared with a Mann-Whitney test. Longitudinal effects on FeLV viral and proviral loads were compared with each other using a Mann-Whitney test carried out with Area Under the Curve (AUC) values. Correlations were assessed using the Spearman test. P-values <0.05 were considered statistically significant.

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Author Contributions

CRT and HL conceived and designed the project; HL supervised the study. CRT and VLR performed the experiments and analyzed the data with the support of VC, MLM and MF. BR was responsible for housing and care of the SPF cats as well as organization and carrying out of blood collections. ME enabled work with Vesicular Stomatitis Virus. BR, RHL, MF, ME, MT and PM provided crucial reagents, materials and/or analysis tools as well as valuable recommendations for their utilization. CRT wrote the manuscript and all other authors critically revised it and approved the final version.

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Figure Captions

Figure 1. ODN 2216 induce proliferation of primary feline immune cells and enhance their expression of costimulatory surface molecules. (A) H^3 thymidine incorporation was assessed in the feline PBMCs of eight adult cats belonging to groups 2 and 3 after stimulation with ODN 2216, ODN 2243 or endotoxin-free PBS. Dots indicate the mean counts per minute (cpm) of triplicate reactions for one cat. **$p<0.01$ (B) Expression of B7.1 surface molecules was assessed 24 hours post stimulation with the indicated treatments. The histogram depicts the fluorescence of gated PBMCs of one cat selected as an example. Isotype control samples are indicated as unstained (left panel). Percentages of positive events in gated PBMCs, lymphocytic (lc) and non lymphocytic (non-lc) subpopulations of cells stimulated with ODN 2216 and ODN 2243 were normalized to percentage of events within the same gates after PBS stimulation (set to 100%). Results for eight cats belonging to groups 2 and 3 are shown (right panel). Stimulation with ODN 2216, ODN 2243 and PBS was compared using Wilcoxon signed

rank tests performed on logarithmic values corresponding to percentages of positive events from the indicated cell populations of eight cats. p values are depicted in the table. (C) Expression of MHCII surface molecules was assessed 24 hours post stimulation with the indicated treatments. The histogram depicts the fluorescence of gated PBMCs of one cat selected as an example (left panel). Mean fluorescence intensity (MFI) of gated PBMCs, lc and non-lc subpopulations of cells stimulated with ODN 2216 and ODN 2243 were normalized to MFI of cells within the same gates after PBS stimulation (set to 100%). Results for eight cats are shown (right panel). Stimulation with ODN 2216, ODN 2243 and PBS was compared using Wilcoxon signed rank tests performed on logarithmic values corresponding to MFI of the indicated cell populations of eight cats. p values are depicted in the table.

Figure 2. ODN 2216 influence IFNα and IL-6 expression in primary feline immune cells. mRNA expression factors of IFNα and IL-6 were measured in (A) fcwf-4 cells stimulated with ODN 2216 and (B, C) feline PBMCs from one cat belonging to group 2 stimulated with either ODN 2216 or control ODN 2243. The transcription of both genes was assessed either 24 hours after treatment of the cells with increasing concentrations of ODN (A left panel and B), or over time after a single stimulation with 4μg/ml ODN (A right panel and C).

Figure 3. ODN 2216 induces an antiviral gene expression profile in PBMCs of adult cats. (A) mRNA expression factors of the indicated genes were measured in PBMCs isolated from fourteen cats belonging to all four age groups and stimulated with ODN 2216 for 24 hours. (B) mRNA expression factors of the indicated IFNα subtype genes

were measured in PBMCs isolated from four adult cats (group 2) and stimulated with ODN 2216 for 24 hours. (C) mRNA levels of TLR9 were measured by real-time qPCR in unstimulated PBMCs of all four groups of cats and normalized to the expression of a feline housekeeping gene (GAPDH) (top panel). Relative TLR9 mRNA expression factors were measured in ODN 2216 stimulated PBMCs from cats of all four groups (bottom panel). $p<0.05$, Perf=perforin, GranB=granzyme B

Figure 4. ODN 2216 induces an antiviral state both in stimulated PBMCs directly and in target cells incubated with supernatants from stimulated PBMCs. (A) mRNA expression factors of the indicated genes were measured in PBMCs of the individual cats (c01-c14) from four different age groups after stimulation with ODN 2216 for 24 hours. (B) Mx mRNA expression factors were assessed at the indicated time points in PBMCs of one cat after a single stimulation with either ODN 2216 or ODN 2243. (C) Mx mRNA expression factors were measured in fcwf-4 cells incubated for 24 hours with supernatants (Sup 2216, Sup 2243, Sup Neg) derived from PBMCs of eight adult cats (groups 2 and 3) or 100U recombinant feline IFNα (rfeIFNα) (left panel). Mx protein was detected by Western blot in fcwf-4 cells incubated with the indicated supernatants derived from PBMCs of one cat belonging to group 2 or with 100U recombinant feline IFNα (rfeIFNα) for 24 hours (right panel). (D) Mx mRNA expression factors were measured in fcwf-4 cells at the indicated time points after a single stimulation with Sup 2216, Sup 2243 and Sup Neg respectively (left panel). Mx protein was detected by Western Blot in fcwf-4 cells at indicated time points after stimulation with Sup 2216 derived from PBMCs of the same cat (right panel). $**p<0.01$

rfeIFNα = recombinant feline IFNα

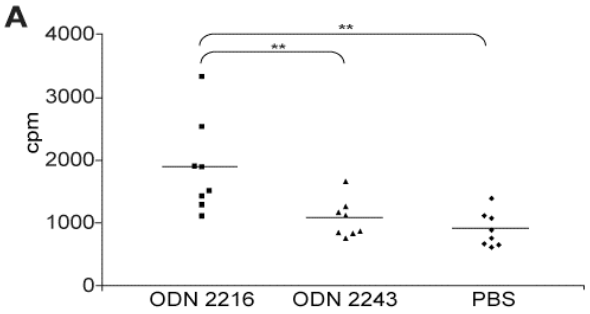
Figure 5. Supernatants derived from ODN 2216 stimulated PBMCs inhibit viral replication in target cells. (A) fcwf-4 cells were incubated for 24 hours with the indicated supernatants derived from PBMCs of eight adult cats (groups 2 and 3) or medium only as control before inoculation with the indicated viruses. Each dot represents mean optical density (OD) values from spectrophotometric readings of plaque assays conducted on duplicate wells treated with supernatants from an individual cat. (B) Correlation of individual inhibition ratios of each virus with Mx mRNA expression induced in fcwf-4 cells incubated with supernatants of ODN 2216 stimulated PBMCs from the eight cats of groups 2 and 3. Note the different scale on the x-axis for each graph indicating the differences in the inhibitory effects of these supernatants on the different viruses. (C) fcwf-4 cells were incubated for 24 hours with the indicated supernatants derived from PBMCs of 4 kittens (group 1) or medium only as control before inoculation with VSV (left panel) or FCV (right panel). Each dot represents mean OD values from spectrophotometric readings of plaque assays conducted on duplicate wells treated with supernatants from one cat. **$p<0.01$

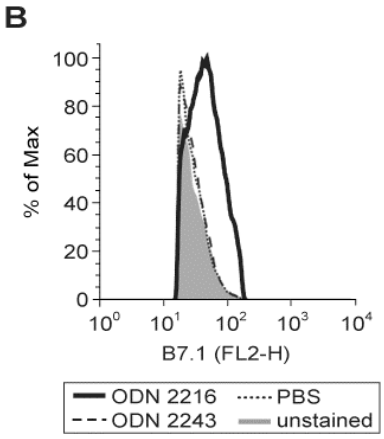
VSV = vesicular stomatitis virus, FCV = feline calicivirus, FPV = feline parvovirus, FCoV = feline coronavirus, FHV = feline herpes virus

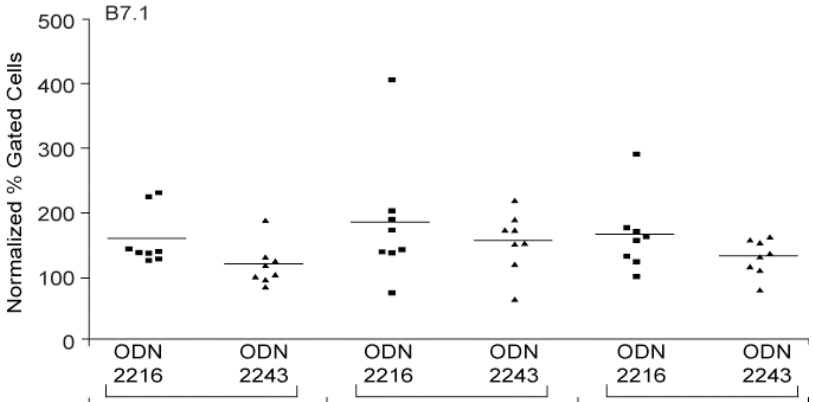
Figure 6. Supernatants derived from ODN 2216-stimulated PBMCs decrease retroviral DNA and RNA loads in target cells. (A) FEA cells were incubated for 24 hours with the respective supernatants or medium alone, before inoculation with the feline leukaemia virus (FeLV), as well as every 2 days thereafter. Viral RNA loads were measured at the indicated time points by real time RT-PCR (top panel). FeLV DNA loads

in the cells were measured at the indicated time points and Ct values were normalized to detection of a housekeeping gene (GAPDH) (bottom panel). Mean values from duplicate experiments carried out simultaneously with the supernatants derived from PBMCs of a selected cat (belonging to group 2) and with medium alone are shown as an example. Results are indicative of those obtained with supernatants from PBMCs of two additional adult cats (from group 3). Stars represent statistical differences in area under the curve (AUC) measurements between the curves of all three cats obtained in cells incubated with Sup 2216 and each of the other treatments. (B) Mx mRNA expression factors (top panel), viral loads (bottom left panel) and proviral loads (bottom right panel) were measured in the FEA cells of five cats (two from group 2 and three from group 3) on day 8 post inoculation. Each dot represents the mean of duplicate measurements for an individual cat. *$p<0.05$, **$p<0.01$

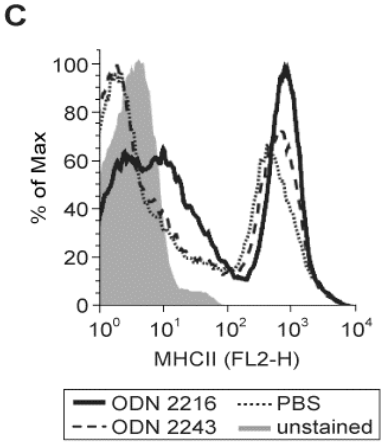
Figure 1

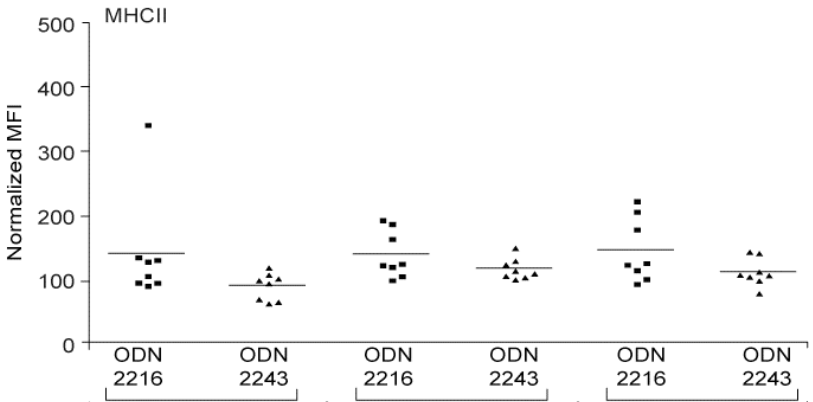






	Non-Lc	Lc	PBMCs
ODN 2216 vs ODN 2243	p=0.0039	p=0.0117	p=0.0078
ODN 2216 vs PBS	p=0.0039	p=0.0117	p=0.0039
ODN 2243 vs PBS	p=0.0977	p=0.0117	p=0.0195





	Non-Lc	Lc	PBMCs
ODN 2216 vs ODN 2243	p=0.0078	p=0.0156	p=0.0156
ODN 2216 vs PBS	p=0.1250	p=0.0078	p=0.0078
ODN 2243 vs PBS	p=0.0977	p=0.0117	p=0.2500

Figure 2

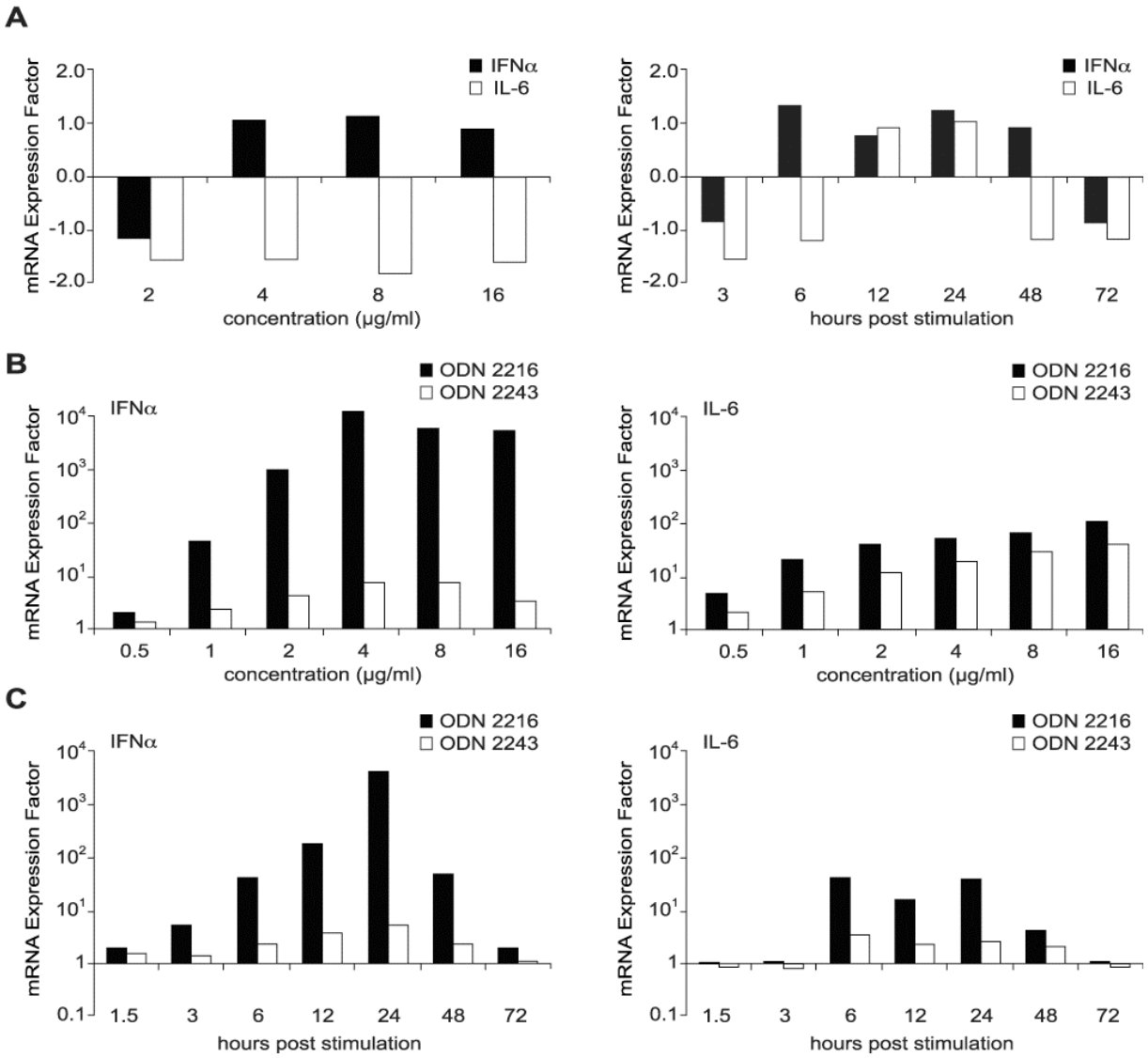

A
IFNα
IL-6
mRNA Expression Factor
2.0
1.0
0.0
-1.0
-2.0
2
4
8
16
concentration (µg/ml)
3
6
12
24
48
72
hours post stimulation
B
ODN 2216
ODN 2243
IFNα
IL-6
10^4
10^3
10^2
10^1
1
0.5
1
2
4
8
16
concentration (µg/ml)
C
0.1
1.5
3
6
12
24
48
72
hours post stimulation

Figure 3

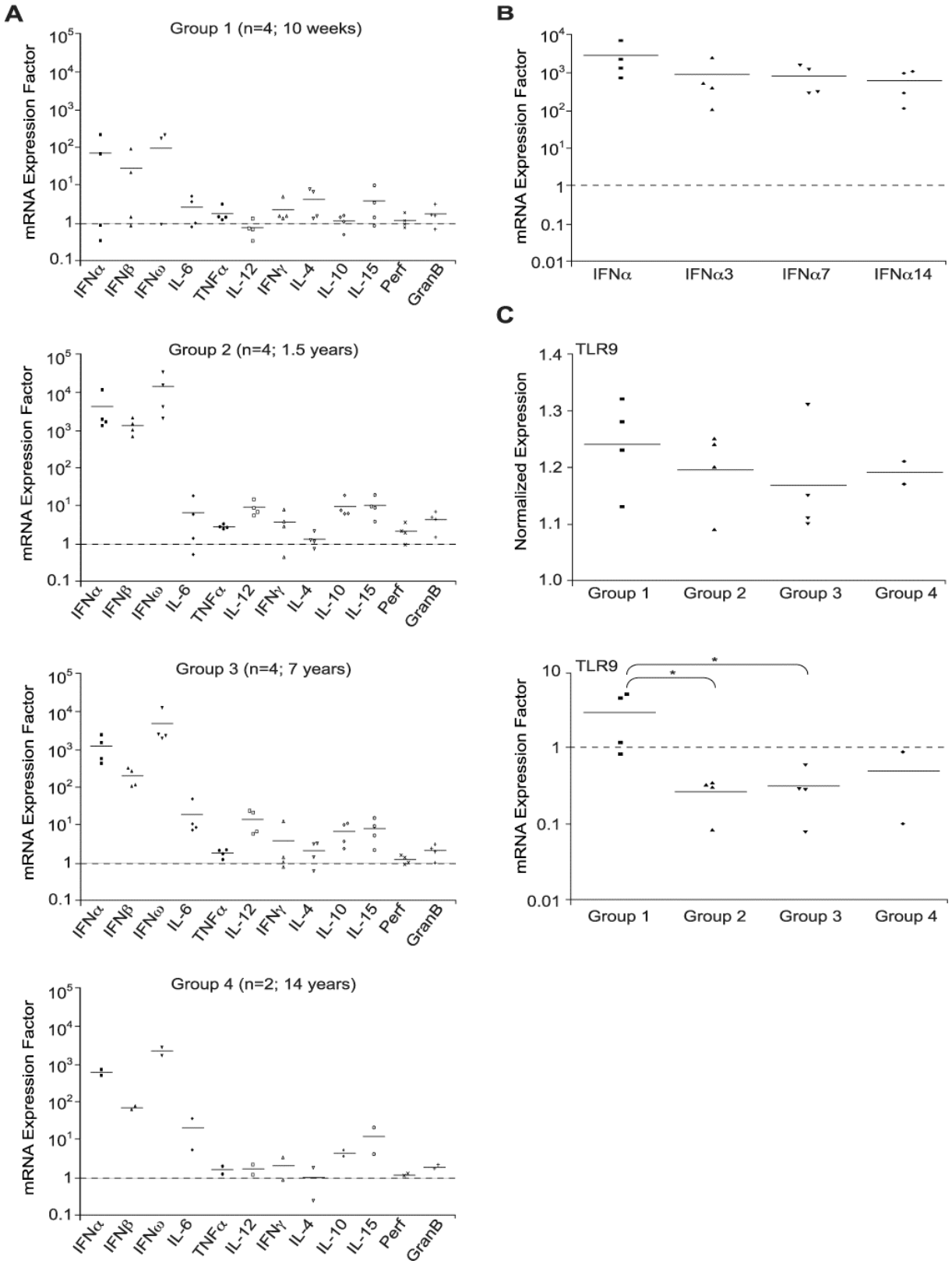

A
Group 1 (n=4; 10 weeks)
Group 2 (n=4; 1.5 years)
Group 3 (n=4; 7 years)
Group 4 (n=2; 14 years)
mRNA Expression Factor
IFNα
IFNβ
IFNω
IL-6
TNFα
IL-12
IFNγ
IL-4
IL-10
IL-15
Perf
GranB
B
mRNA Expression Factor
IFNα
IFNα3
IFNα7
IFNα14
C
TLR9
Normalized Expression
Group 1
Group 2
Group 3
Group 4
TLR9
mRNA Expression Factor
*
*

Figure 4

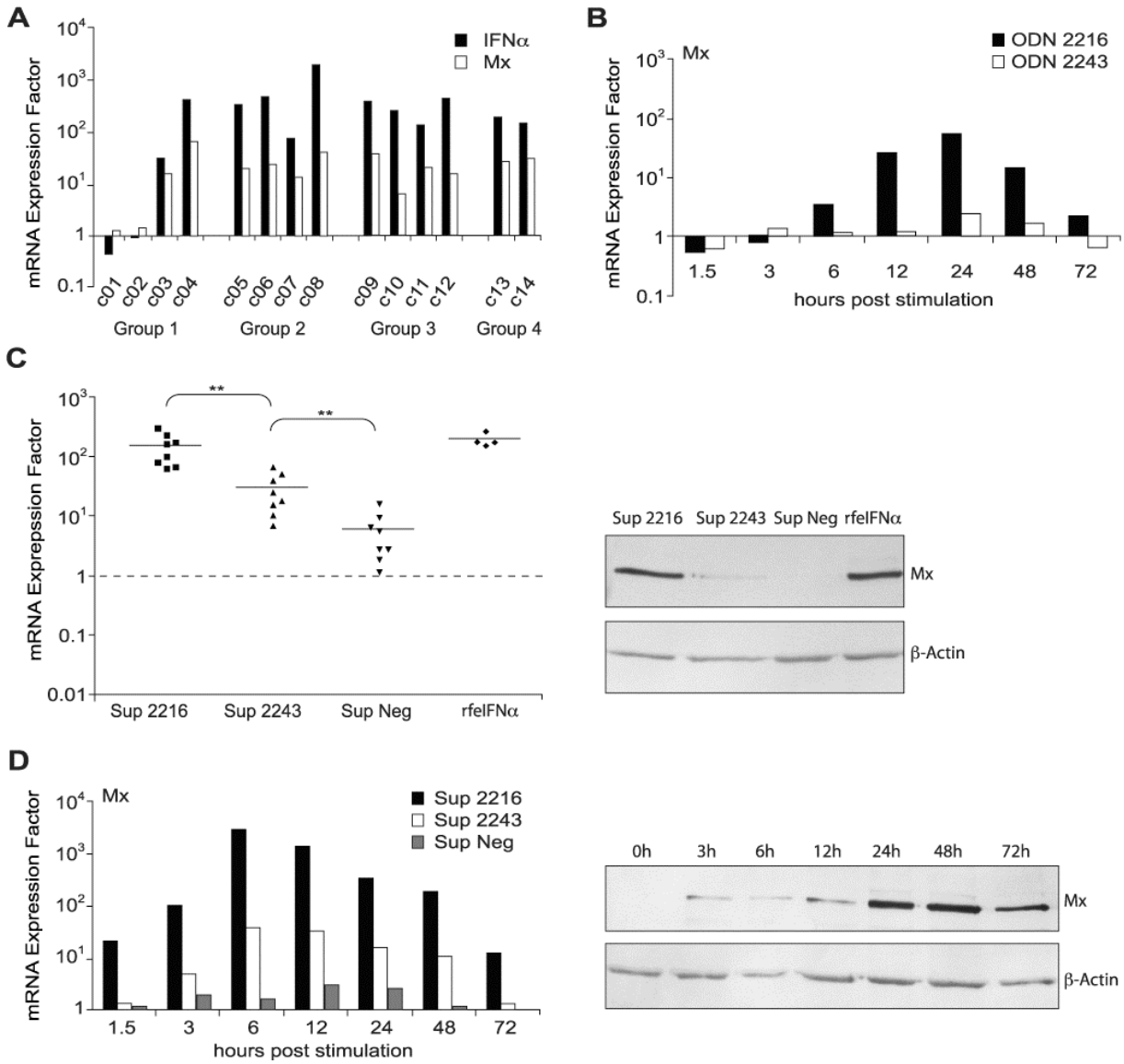

A
mRNA Expression Factor
IFNα
Mx
c01 c02 c03 c04 c05 c06 c07 c08 c09 c10 c11 c12 c13 c14
Group 1
Group 2
Group 3
Group 4
B
Mx
ODN 2216
ODN 2243
mRNA Expression Factor
1.5 3 6 12 24 48 72
hours post stimulation
C
mRNA Exprepssion Factor
**
**
Sup 2216
Sup 2243
Sup Neg
rfeIFNα
Sup 2216 Sup 2243 Sup Neg rfeIFNα
Mx
β-Actin
D
Mx
Sup 2216
Sup 2243
Sup Neg
mRNA Expression Factor
1.5 3 6 12 24 48 72
hours post stimulation
0h 3h 6h 12h 24h 48h 72h
Mx
β-Actin

Figure 5

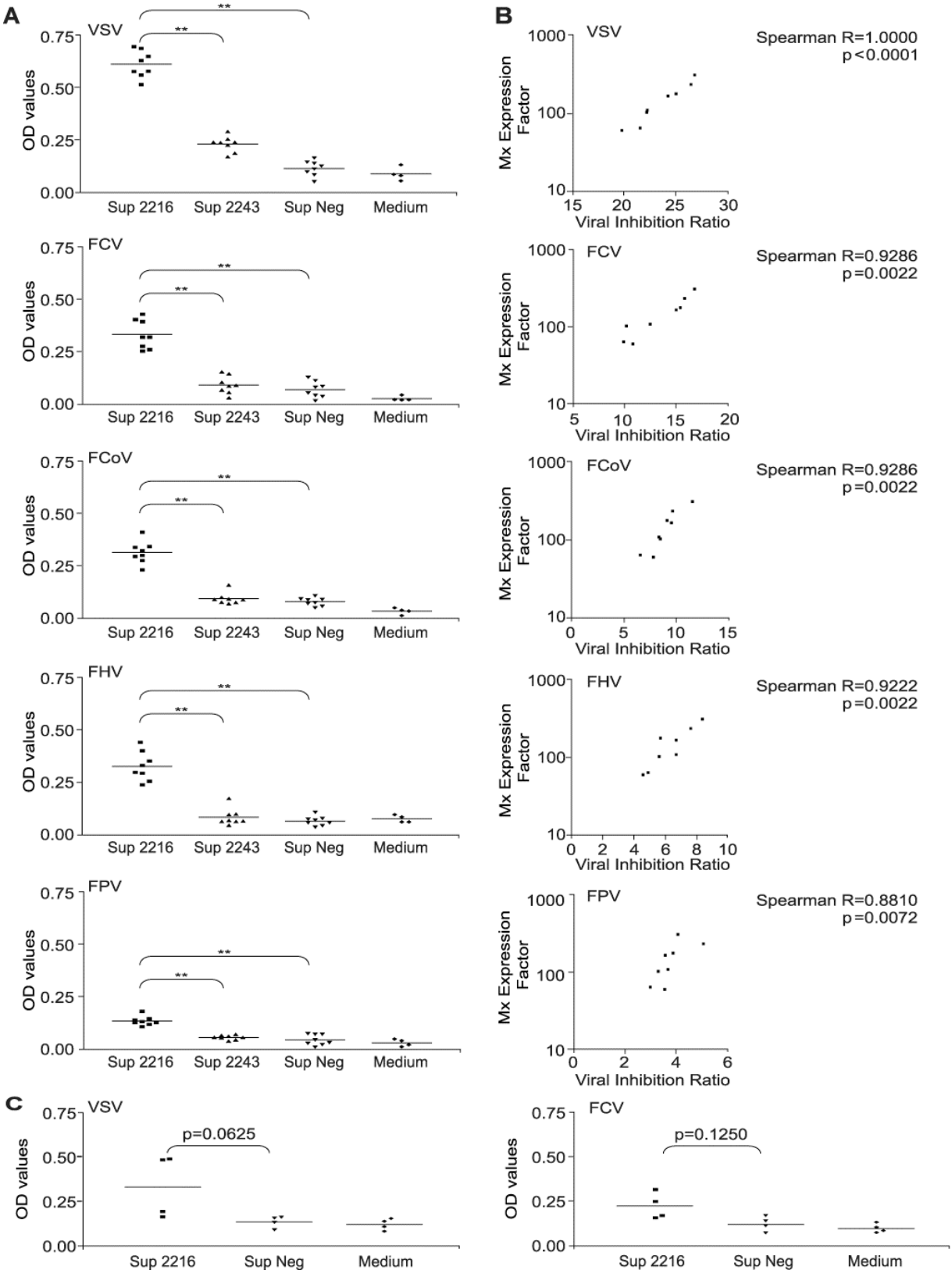
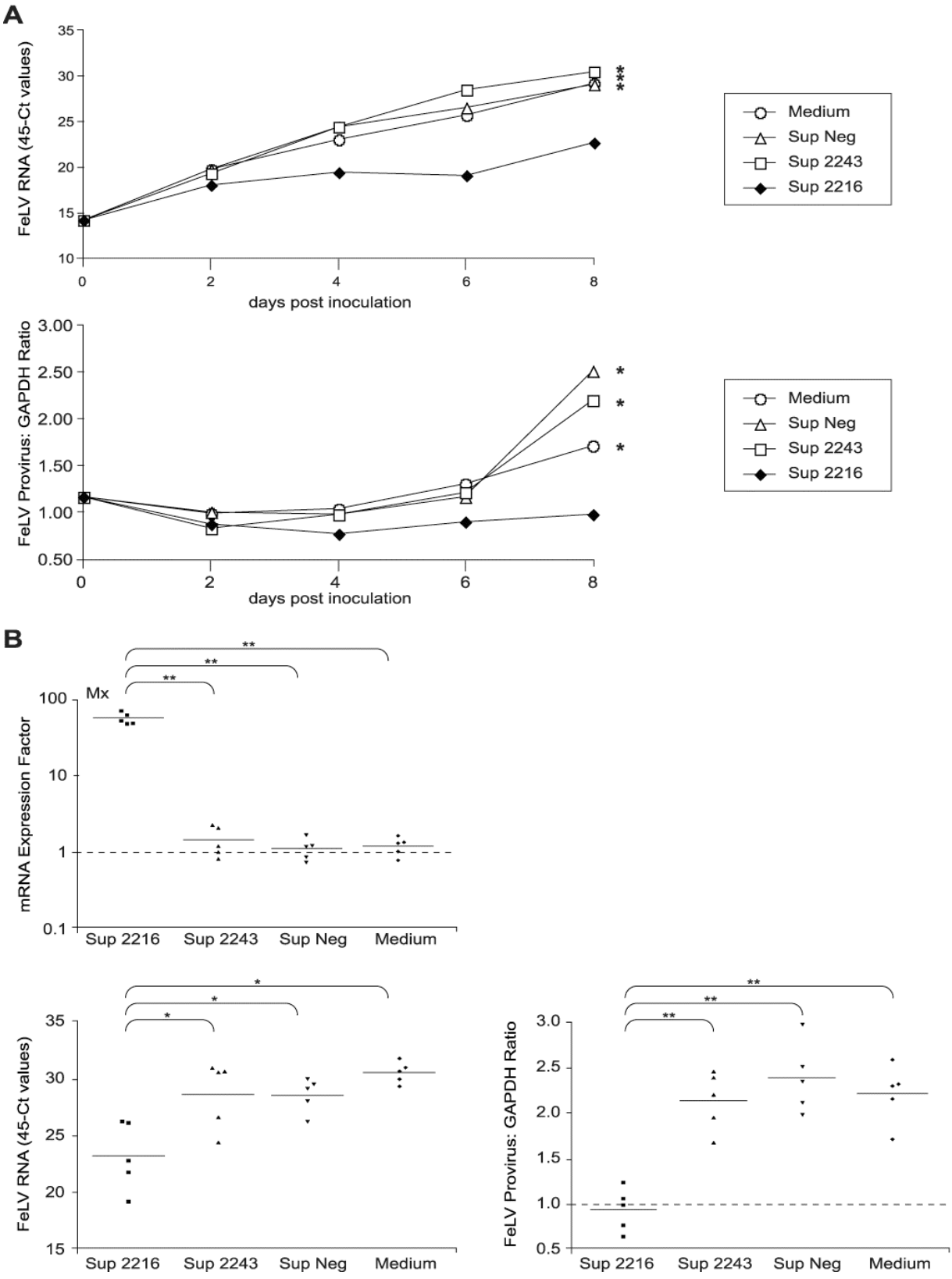

A
VSV
FCV
FCoV
FHV
FPV
OD values
0.75
0.50
0.25
0.00
**
Sup 2216
Sup 2243
Sup Neg
Medium
B
Mx Expression Factor
1000
100
10
Viral Inhibition Ratio
Spearman R=1.0000
p<0.0001
Spearman R=0.9286
p=0.0022
Spearman R=0.9286
p=0.0022
Spearman R=0.9222
p=0.0022
Spearman R=0.8810
p=0.0072
C
p=0.0625
p=0.1250

Figure 6


A
FeLV RNA (45-Ct values)
days post inoculation
Medium
Sup Neg
Sup 2243
Sup 2216
FeLV Provirus: GAPDH Ratio
days post inoculation
Medium
Sup Neg
Sup 2243
Sup 2216
B
Mx
mRNA Expression Factor
Sup 2216
Sup 2243
Sup Neg
Medium
FeLV RNA (45-Ct values)
Sup 2216
Sup 2243
Sup Neg
Medium
FeLV Provirus: GAPDH Ratio
Sup 2216
Sup 2243
Sup Neg
Medium

4. Addendum

Additional data that was accumulated during the course of this project but either not incorporated or not illustrated ("data not shown") in the manuscripts of chapter 3, will be presented in this chapter. While chapter 4.1 briefly describes materials and methods used for the experiments illustrated herafter, each section of chapter 4.2 comprises a short explanation regarding the aim of the experiments accompanied by figures and captions. When indicated, information concerning the localization of corresponding explanations or data in the manuscripts of chapter 3 will be provided. The figures and table included in this section are numbered 4.1 to 4.10. The results of this chapter will be discussed separately in section 4.4.

4.1 Material and methods

Unless specified otherwise, all materials and methods utilized to generate the data presented in this chapter, including the animals comprised in the study, the experimental set-ups, cell culture conditions, IRM concentrations utilized for stimulation, relative gene expression analyses, amounts of cells loaded in Western Blot samples, production of supernatants as well as viral concentrations and carrying out of viral inhibition assays are described precisely in the corresponding "material and methods" sections of both manuscripts of section 3. Importantly, the method utilized for the calculations of relative mRNA expression factors illustrated in many graphs of this section can be founding the "material and methods" section of manuscript 2, under "relative gene expression analysis". The appropriate manuscript section that confers additional information to the figures in this section will be referred to in each part of chapter 4.2.

4.2 Innate immune properties of feline cell lines

4.2.1 Expression of TLRs by feline cell lines

The IRMs utilized were selected for their known affinity to specific TLRs. Indeed, the binding of Poly IC to TLR3, R-848 to TLRs 7 and 8, and dSLIM™ and ODN 2216 to TLR9 is known to initiate cascades of events that eventually lead to the transcription of genes belonging to the proinflammatory and type I IFN families. In determining whether

the chosen IRMs may successfully stimulate feline cells, the first step was to establish whether feline cells expressed these TLRs. CrFK, FEA and fcwf-4 cells, as well as purified PBMCs were thus tested for their basal expression of TLRs 3, 7, 8 and 9 when left unstimulated in culture. Experiments were carried out as described in the materials and methods section of manuscript 2, under "relative gene expression analysis". Furthermore TLR9 expression in PBMCs of fourteen cats belonging to four different age groups can be seen in Fig 3C of manuscript 2.

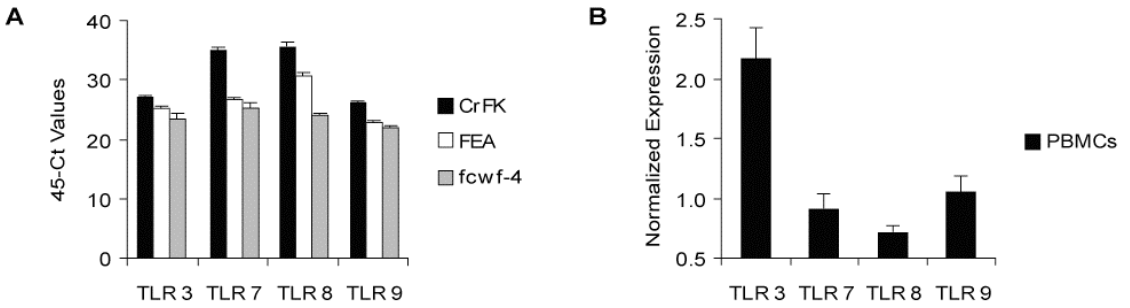


Fig 4.1. Toll-like receptor expression in feline cells.
(A) mRNA expression of the indicated *tlr* genes was measured by real-time qPCR in 10^5 unstimulated cells of the indicated cell lines. 45 cycles-cycle threshold (Ct) values were calculated and means of triplicate wells are depicted. (B) mRNA levels of TLR9 were measured by real-time qPCR in unstimulated PBMCs of one cat and normalized to the expression of a feline housekeeping gene (GAPDH). Values represent means of triplicate wells. Results are representative of at least two experiments.

4.2.2 IRM stimulation of feline cell lines

Although stimulation of purified feline PBMCs with various IRMs effectively altered the gene profile measurable in these cells (Fig. 1A-C of manuscript 1 and Fig. 2B-C of manuscript 2), immortalized feline cell lines remained largely insensitive to treatment with the same IRMs. A series of experiments were initially carried out on cell lines of both epithelial (CrFK) and fibroblastic (FEA and fcwf-4) origins to determine whether incubation with IRMs would influence the expression of genes related to innate immunity in these cells. The effects linked to both concentration and duration of incubation with each IRM were tested, and the expressions of IFNα and IL-6 genes were selected as readouts for influence on innate immune parameters.

All three cell lines reacted similarly to incubation with the different IRMs: the selected genes remained largely unaffected regardless of the amount of IRM the cells were subdued to and the duration of incubation. Altogether, considering all IRMs tested and all

cell lines utzilized, the relative expression of both IFNα and IL-6 genes maximally decreased upon stimulation by 1.8 fold and maximally increased by 1.6-fold.

As ODN 2216 clearly indicated the strongest induction of innate immune parameters in feline PBMCs when compared to Poly IC, R-848 and dSLIM™ (consider Fig. 1A-C of manuscript 1 and Fig. 2B-C of manuscript 2), only the effects of this IRM on various feline cell lines will be shown here. The observations illustrated below for CrFK and FEA cells confirm the results for fcwf-4 cells that can be consulted in Fig. 2A of manuscript 2. Precise information on methods utilized for the *in vitro* stimulation of the cells and the determination of relative expression of IFNα and IL-6 are given in the "material and methods" section of manuscript 2, under "relative gene expression analyis". The IRM concentrations and incubation times tested were identical for all IRMs, and are indicated in the figure below (Fig. 4.2).

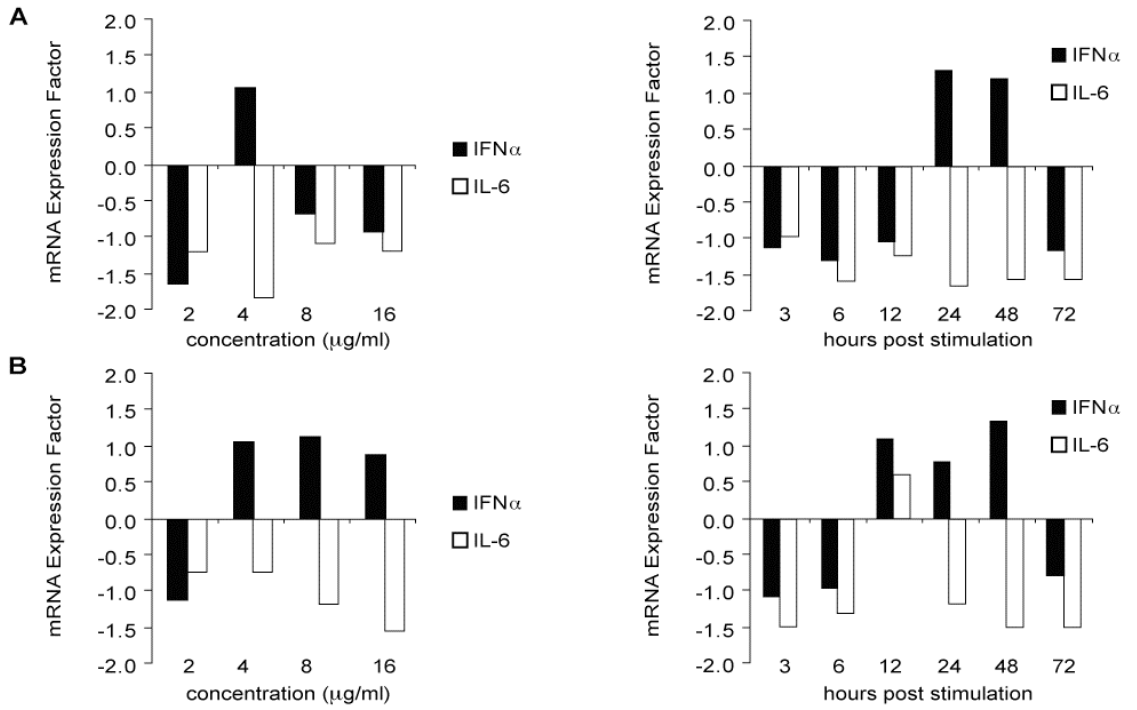


Fig 4.2. Feline cell lines remain uninfluenced by the direct stimulation with IRMs

mRNA expression factors of *ifnα and il-6* genes were measured in CrFK (A) and FEA (B) cells stimulated with ODN 2216. The transcription of both genes was assessed either 24 hours after treatment of the cells with increasing concentrations of ODN (left panels), or over time after a single stimulation with 4μg/ml ODN (right panels). Depicted are mean expression factors calculated from duplicate experiments carried out simultaneously.

4.3 Immunomodulation by ODN 2216

The experiments described in this section aimed at completing the evaluation of the immunomodulatory and antiviral properties of ODN 2216 thoroughly described in manuscript 2. Experiments aiming to analyze the induction of both Mx expression (as described in section 4.3.1) and viral inhibition (described in section 4.3.2) in target cells by other IRMs were also partially conducted, however with insignificant success. These experiments will thus not be illustrated here. The methods linked to this section are further fully described in the corresponding section of manuscript 2, under "production of supernatants", relative gene expression analysis, "Western blot" and "viral inhibition assay".

4.3.1 Induction of Mx expression in feline target cells

Stimulation of feline PBMCs with ODN 2216 enhanced the expression of a wide array of genes, among which many subtypes of type I IFN (Fig 3A-B, manuscript 2). This led to assume that supernatants of stimulated PBMCs most probably contained a broad variety of type I IFN subtypes, which should be capable of initiating the production of intracellular antiviral proteins in target cells, providing these cells express an IFN receptor. In order to determine whether and to which extent the immortalized feline cell lines used in this study have conserved the capability to respond to type I IFN stimulation, the induction of expression of the Mx gene was measured in these cells after stimulation with the rfeIFNα protein. CrFK, FEA and fcwf-4 cells all responded to rfeIFNα stimulation, however with different sensitivities: while 10U rfeIFNα sufficed to induce a 60 respectively 200-fold increase in Mx mRNA in FEA and fcwf-4 cells, 100U were required to attain comparable transcription levels in CrFK cells (Fig. 4.3)

Thus, as the IRMs did not confer feline cell lines measurable alterations upon direct stimulation (see section 4.2.2), the antiviral potential of ODN 2216 on these cells was measured indirectly: supernatants derived from feline PBMCs stimulated with ODN 2216 (or ODN 2243 or endotoxin-free PBS as controls), were incubated with a target cell line in which the induction of the Mx gene was then analyzed. This method was also useful for later viral inhibition experiments, as these cell lines are susceptible to different feline viruses, while purified PBMCs are not. Large quantities of supernatants were produed from the PBMCs purified from the blood of several SPF cats, so that their use in

parallel experiments conducted on different cell lines would confer comparable results. Also, since the antiviral properties conferred by the supernatants on different cell lines could only be effectively compared if the target cells were susceptible to the same viruses, two sets of experiments were conducted.

In a first series of experiments, supernatants were produced with isolated PBMCs from eight adult cats (four of 1.5 and four of 7 years of age) and four kittens (10 weeks of age). With the aim to determine not only the the capability of these supernatants to induce antiviral mechanisms (see experiments shown in this section and manuscript 2, Fig. 4C-D) but also to inhibit viral replication in target cells (see section 4.3.2 and Fig. 5 of manuscript 2), they were then utilized to stimulate separate cultures of both CrFK and fcwf-4 cells, as FCV, FHV, FCoV and FPV can replicate in both these cell lines and induce observable cytopathic effects (CPE). Although the results obtained for fcwf-4 cells are thoroughly presented in manuscript 2, somewhat modified graphs are shown in this section to facilitate comparisons with CrFK cells.

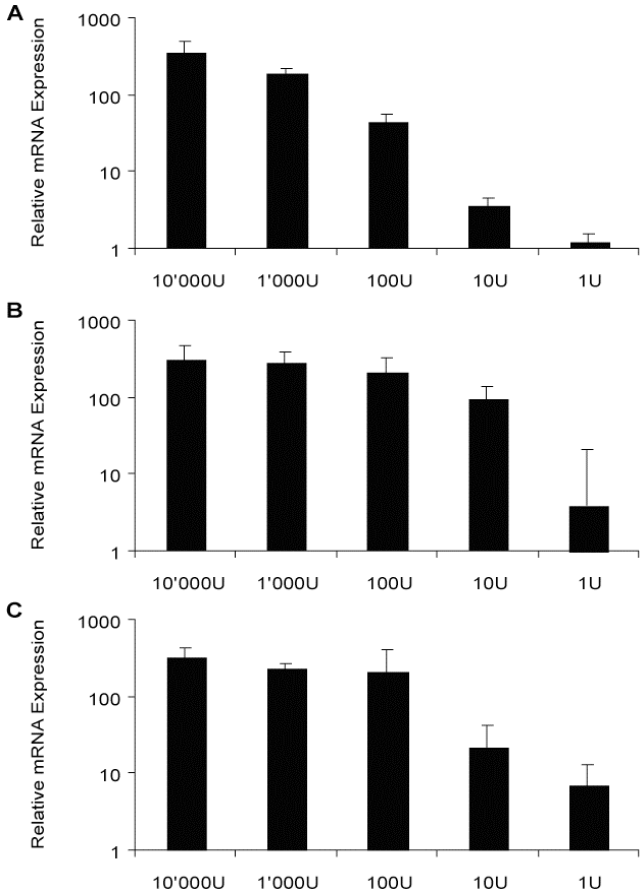


Fig. 4.3. Sensitivity of feline cell lines to recombinant feline IFNα
mRNA expression factors of the *mx* gene were measured in CrFK (A), fcwf-4 (B) and FEA (C) cells stimulated for 24 hours with the indicated amounts of rfeIFNα. Depicted are mean expression factors calculated from triplicate experiments carried out simultaneously.

The expression of type I IFN genes in the ODN 2216-stimulated PBMCs of individual cats highly correlated with the induction of Mx in target cells by the supernatants (manuscript 2, Fig. 4A). Thus, as the PBMCs of all adult cats increased their type I IFN mRNA expression upon stimulation with ODN 2216 by up to 12'000 fold (manuscript 2, Fig. 3A), the supernatants derived from these immune cells significantly induced Mx expression in target CrFK and fcwf-4 cells (Fig. 4.4). In the same sense, the Mx mRNA levels remained unaltered when both cell lines were treated with supernatants derived from the PBMCs of two kittens that failed to respond to ODN 2216 stimulation (Fig 4.5). Supernatants of PBMCs treated with the control ODN 2243 could only induce Mx mRNA expression accompanied by slightly detectable Mx protein expression in fcwf-4 cells, the most sensitive cell line to rfeIFN. Direct stimulation of the cells with ODN 2216 had notably no effect on the expression of Mx in these cells.

In accordance with the weaker sensitivity of CrFK to rfeIFNα, the induction of Mx in these cells appeared weaker than in fcwf-4 cells, not only on the mRNA level but also when considering proteins, as can be interpreted by the mRNA expression factors and the proportional differences in the Mx and β-actin bands depicted in Fig. 4.4. Actually, the mean fold induction in Mx mRNA by the supernatants of eight adult cats in both cell lines enable to estimate the amount of supernatant applied in the cell culture experiments: supernatants derived from PBMCs stimulated with ODN 2216 appear to contain close to 100U of type I IFN; consequently, when 100U rfeIFN were applied to the cultures of both cell lines, the mean increase of Mx mRNA measured in four wells was highly similar to that induced by the ODN-2216 derived supernatants of the eight adult cats (Fig 4.4).

Further experiments with these supernatants enabled to observe the kinetics of Mx expression in both cell lines. When the target cells were harvested at regular time points after treatment with the supernatant of one adult cat, it became evident that Mx protein appears with some delay when compared to its mRNA. While the highest mRNA expression for the Mx gene was in both cell lines around 6 hours post stimulation, Mx protein peaked after 24 hours in fcwf-4 cells, and seemed to remain stable between 6 and 72 hours in CrFK cells (Fig. 4.6). The proportional protein expressions of Mx compared to β-actin as estimated by the density of the bands in each experiment again support much stronger Mx expression by fcwf-4 cells.

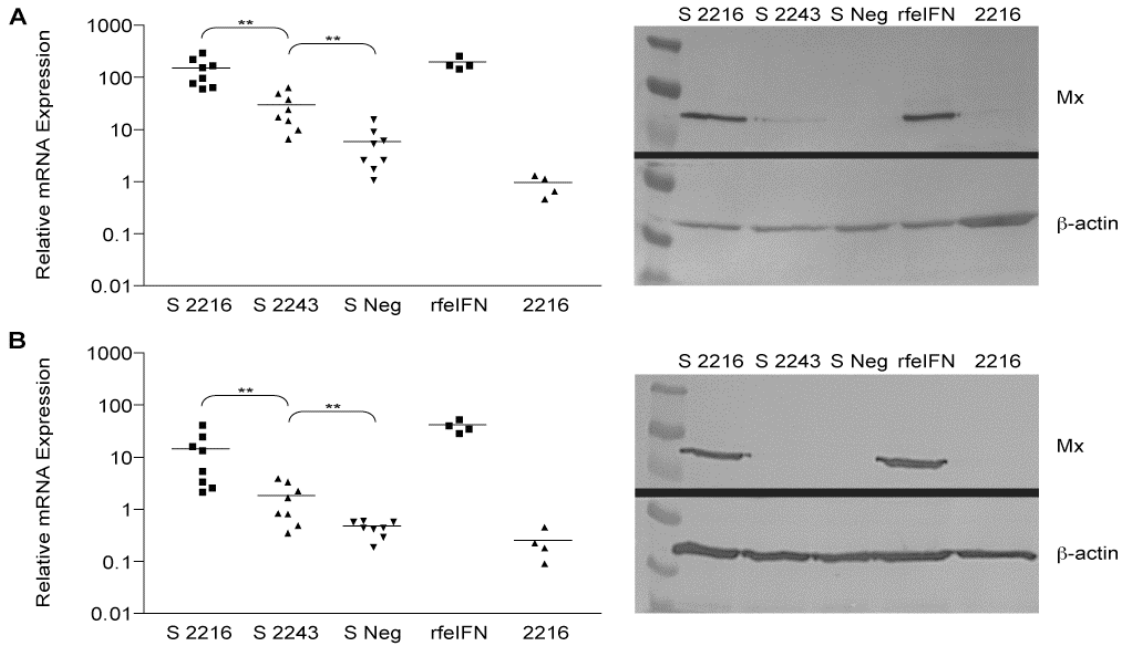


Fig 4.4. Induction of Mx expression in fcwf-4 and CrFK cells by supernatants derived from ODN 2216-derived PBMCs of adult cats

Mx mRNA (left panels) and protein (right panels) levels were measured by real-time qPCR and by Western blot respectively in fcwf-4 (A) and CrFK (B) cells. mRNA expression factors and were measured in the cells after incubation for 24 hours either with supernatants derived from PBMCs stimulated with ODN 2216 (S 2216), ODN 2243 (S 2243) and endotoxin-free PBS (S Neg), or with 100U recombinant feline IFNα (rfeIFNα), or with ODN 22126 (2216) directly. Supernatants from 8 adult cats were used. Each symbol represents mean values for duplicate measurements carried out simultaneously with the supernatants from the PBMCs of one cat. Mx protein was detected by Western blot in the cells after incubation with either the indicated supernatants derived from PBMCs of one cat, or with 100U rfeIFNα, or with ODN 2216 directly. **p<0.01

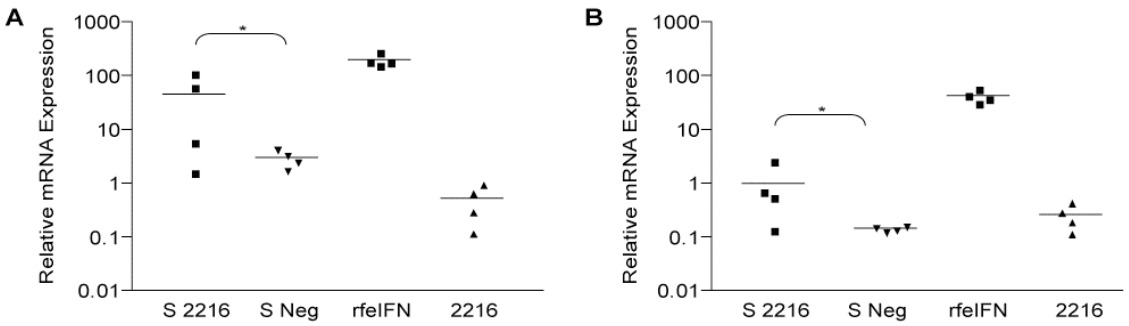


Fig 4.5. Induction of Mx expression by supernatants derived from ODN 2216-stimulated PBMCs of four kittens

mRNA expression factors and were measured in fcwf-4 (A) and CrFK (B) cells after incubation for 24 hours either with supernatants derived from the PBMCs stimulated with ODN 2216 (S 2216), ODN 2243 (S 2243) and endotoxin-free PBS (S Neg), or with 100U recombinant feline IFNα (rfeIFNα), or with ODN 22126 (2216) directly. Supernatants from 4 kittens were used. Each symbol represents mean values for duplicate measurements carried out simultaneously with the supernatants from the PBMCs of an individual cat.* p<0.05

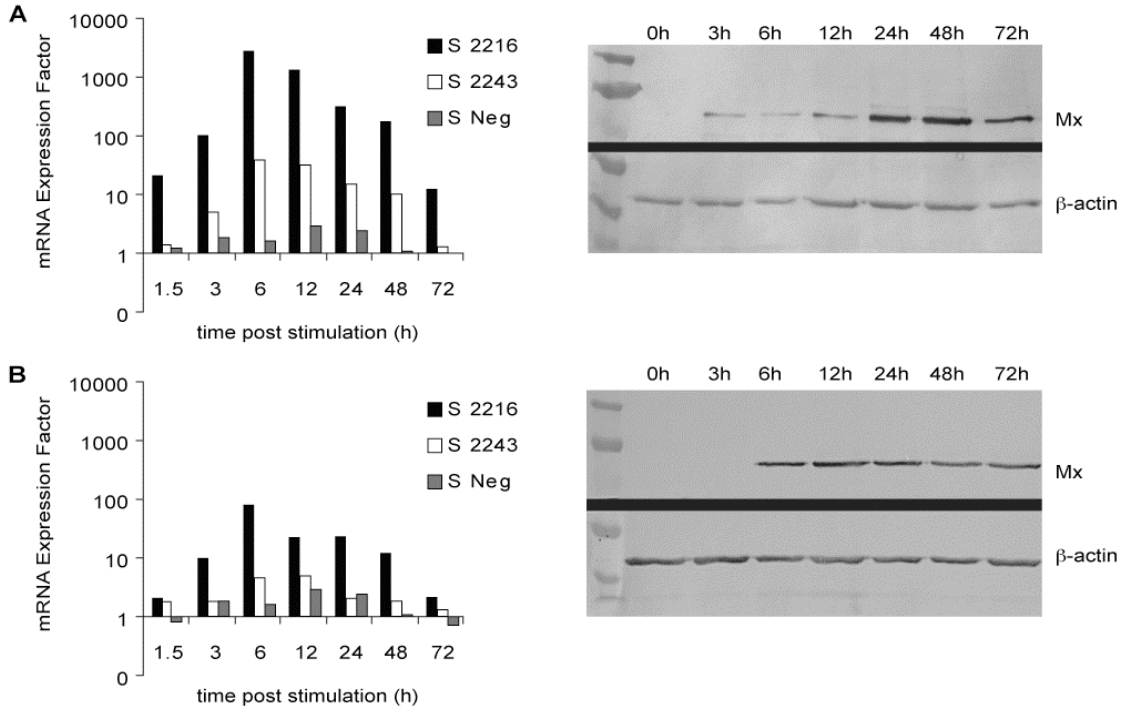


Fig 4.6. Kinetics of Mx expression after stimulation of target cells with supernatants derived from ODN 2216-stimulated PBMC

Mx mRNA (left panels) and protein (right panels) levels were measured by real-time qPCR and by Western blot respectively in fcwf-4 (A) and CrFK (B) cells. mRNA expression factors were measured in the cells at the indicated time points after a single stimulation with supernatants derived from PBMCs stimulated with ODN 2216 (S 2216), ODN 2243 (S 2243) or endotoxin-free PBS (S Neg). Supernatants from one cat were used. Depicted are mean expression factors calculated from duplicate experiments carried out simultaneously.

Mx protein was detected by Western Blot in the cells at the indicated time points after stimulation with S 2216 derived from PBMCs of the same cat than above.

In a second series of experiments, new supernatants were produced by stimulation of purified PBMCs from five adult cats with ODN 2216 and controls ODN 2243 and endotoxin-free PBS. The cells of these five animals had already been used in the first experiment described above, however, the experiments cannot be directly compared, since the blood was collected from the cats at a different time point. These new supernatants were incubated with FEA cells, and were predestined to be used in experimental inhibition of FeLV, as this virus effectively replicates in this cell line. While direct stimulation of these cells with ODN 2216 did not induce any alterations in the expression of Mx, the supernatants derived from ODN 2216-stimulated PBMCs induced high levels of Mx mRNA in FEA cells, in concordance with the above-mentioned sensitivity to rfeIFNα of this cell line (Fig 4.7). Direct stimulation with ODN 2216 did not lead affect Mx mRNA expression. However, in contrast to observations in

CrFK and fcwf-4 cells, the supernatants produced by ODN 2243-stimulated PBMCs did not significantly induce Mx expression.

Fig 4.7. Induction of Mx expression in FEA cells by supernatants derived from ODN 2216-derived PBMCs of adult cats

Mx mRNA levels were measured by real-time qPCR in FEA cells. After incubation for 24 hours either with supernatants derived from PBMCs stimulated with ODN 2216 (S 2216), ODN 2243 (S 2243) and endotoxin-free PBS (S Neg), or with 100U recombinant feline IFNα (rfeIFNα), or with ODN 22126 (2216) directly. Supernatants from the PBMCs of 8 adult cats were used. Each symbol represents mean values for duplicate measurements carried out simultaneously with the supernatants from the PBMCs of one cat. ** $p<0.01$, * $p<0.05$

4.3.2 Viral Inhibition in CrFK cells

The inhibition of viral propagation in cultures of fcwf-4 cells by supernatants derived from ODN 2216-stimulated PBMCs was fully reported in manuscript 2. When the cells were incubated, prior to their inoculation, with the supernatants derived from PBMCs of adult cats, not only VSV, the common IFN-sensitive control in such experiments, but also FCV, FHV, FCoV and FPV were repressed (manuscript 2, Fig. 5A). Also, only the supernatants of ODN 2216-stimulated PBMCs of two kittens could also inhibit viral replication in fcwf-4, and with lower efficiency (manuscript 2, Fig 5C). Although not all viruses indicated the same sensitivity to the supernatants, the extent of inhibition of each virus strongly correlated with the induction of Mx in fcwf-4 cells by the individual supernatants (manuscript 2, Fig. 5B). These results corroborate those obtained by the viral inoculation of CrFK cells previously treated with the same supernatants (Fig. 4.8, 4.9). The lower sensitivity of CrFK cells to rfeIFNα is also reflected in these viral inhibition experiments (Tables 4.1 and 4.2) Notably, if the cells were stimulated directly with ODN 2216 in place of supernatants of ODN 2216 treated supernatants, no inhibition of virus could be observed (Fig. 4.8, 4.9). Although not shown in manuscript 2 for fcwf-4 cells, these controls were also carried out and rendered similar results.

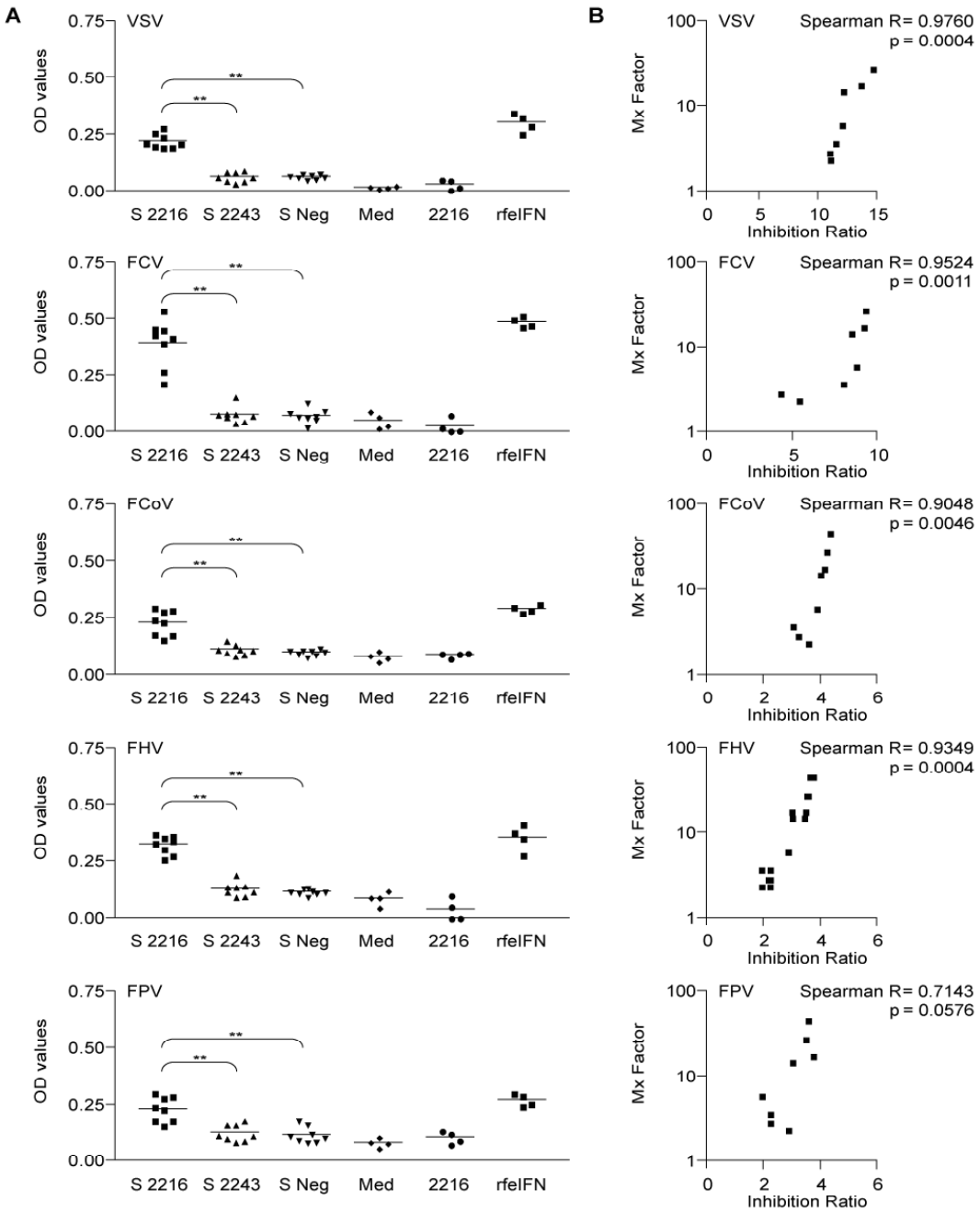


Fig 4.8. Viral inhibition on CrFK cells by supernatants derived from ODN 2216-stimulated PBMCs of adult cats

(A) CrFK cells were incubated for 24 hours with supernatants derived from PBMCs stimulated with ODN 2216 (S 2216), ODN 2243 (S 2243) and endotoxin-free PBS (S Neg), or with medium only, or with ODN 2216 directly (2216) or with 100U recombinant feline IFNα (rfeIFNα), prior to inoculation with the indicated viruses. Supernatants from the PBMCs of 8 adult cats were used. Each dot represents mean optical density (OD) values from spectrophotometric readings of plaque assays conducted on duplicate wells treated with supernatants from an individual cat. (B) Correlation of individual inhibition ratios of each virus with Mx mRNA expression factor (Mx Factor) induced in CrFK cells incubated with supernatants of ODN 2216-stimulated PBMCs from eight adult cats. Note the different scale on the x-axis for each graph indicating the differences in the inhibitory effects of these supernatants on the different viruses. ** $p<0.01$

VSV = Vesicular Stomatitis Virus, FCV = Feline Calicivirus, FPV = Feline Parvovirus, FCoV = Feline Coronavirus, FHV = Feline Herpes Virus

Table 4.1. Means of viral inhibition rates measured in CrFK cells after treatment with supernatants derived from stimulated PBMCs of 8 adult cats

	VSV	FCV	FCoV	FHV	FPV
S 2216	**12.68**	**8.02**	**3.78**	**3.01**	**2.89**
S 2243	**3.65**	**1.55**	**1.50**	**1.43**	**1.55**
S Neg	**3.69**	**1.44**	**1.34**	**1.26**	**1.44**

Depicted are mean viral inhibition rates for 8 cats. Viral inhinition rates for each cat were calculated with the following formula:

Mean optical density (OD) values of duplicate wells treated with Supernatant / Mean OD values of quadruplicate wells treated with medium alone (see materials and methods manuscript 2, "viruses and viral inhibition assays")

S=supernatant derived from PBMCs stimulated for 24 hours with either ODN 2216 (S 2216), ODN 2243 (S 2243) or endotoxin-free PBS (S Neg)

VSV= Vesicular Stomatitis Virus, FCV= Feline Calicivirus, FCoV=Feline Coronavirus, FHV=Feline Herpes Virus, FPV=Feline Parvovirus

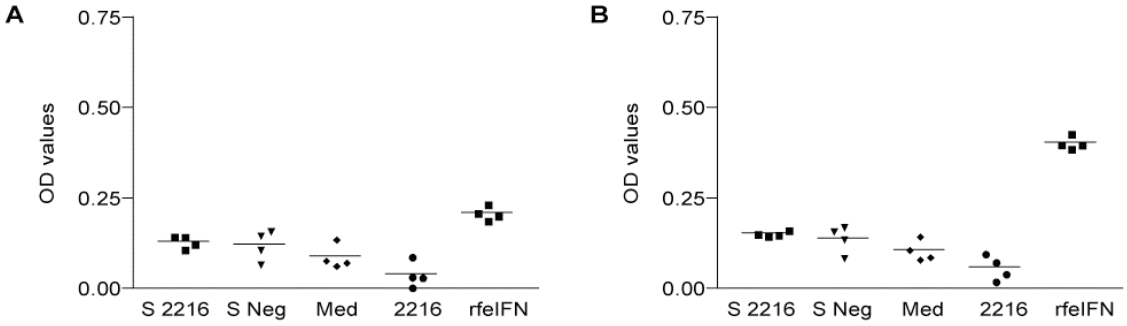


Fig 4.9. Viral inhibition on CrFK cells by supernatants derived from ODN 2216-stimulated PBMCs of kittens

CrFK cells were incubated for 24 hours with supernatants derived from PBMCs stimulated with ODN 2216 (S 2216) or endotoxin-free PBS (S Neg), or with medium only, or with ODN 2216 directly (2216) or with 100U recombinant feline IFNα (rfeIFNα), prior to inoculation with VSV (A) or FCV (B). Supernatants from the PBMCs of 4 kittens were used. Each dot represents mean optical density (OD) values from spectrophotometric readings of plaque assays conducted on duplicate wells treated with supernatants from an individual cat. VSV = vesicular stomatitis virus, FCV = feline calicivirus

4.3.3 Viral Inhibition kinetics

The expression of Mx protein in target cells incubated with supernatants derived from ODN 2216-stimulated PBMCs remains elevated for at least 72 hours (Fig. 4.4). Also, within 24 hours of incubation with these supernatants, the cells acquired significant resistance to the inoculation with many viruses (Fig 4.8). The experiments presented in this section indicate that even after 72 hours incubation with the supernatants, target cells maintain some resistance potential. Due to the insufficient amounts of the supernatants

produced for the analyses above (see sections 4.3.1 and 4.3.2), these experiments were only conducted on fcwf-4 cells and with VSV and FCV (Fig. 4.10).

Table 4.2. Means of viral inhibition rates measured in fcwf-4 and CrFK cells after treatment with supernatants derived from stimulated PBMCs of 4 kittens

	fcwf-4		CrFK	
	VSV	**FCV**	**VSV**	**FCV**
S 2216	**2.69**	**2.20**	**1.47**	**1.43**
S Neg	**1.13**	**1.32**	**1.37**	**1.30**

Depicted are mean viral inhibition rates for 4 cats. Viral inhinition rates for each cat were calculated with the following formula:

Mean optical density (OD) values of duplicate wells treated with Supernatant / Mean OD values of quadruplicate wells treated with medium alone (see materials and methods manuscript 2, "viruses and viral inhibition assays)

S=supernatant derived from PBMCs stimulated for 24 hours with either ODN 2216 (S 2216) or endotoxin-free PBS (S Neg)

VSV=Vesicular Stomatitis Virus, FCV=Feline Calicivirus, FCoV=Feline Coronavirus, FHV=Feline Herpes Virus, FPV=Feline Parvovirus

4.4 Statistics

The statistical tests utilized to determine significance in the experiments of this section are precisely described in the manuscripts of section 3. For clearer understanding of the graphics depicting many different conditions, significance is shown with a star (*) only for those groups considered relevant or not obvious. Additional information is gien in the legends of the figures when required.

4.5 Discussion of additional data

Altogether, the data presented in this section confirm and complete results shown or mentioned in the manuscripts of section 3 of this document. Although CrFK, fcwf-4 and FEA cell lines express the receptors required for the recognition of the selected IRMs, no response to *in vitro* stimulation with these synthetic molecules could be detected with the available tools. Concordantly, no viral inhibition could be observed in these cell lines when they were stimulated with the IRMs directly.

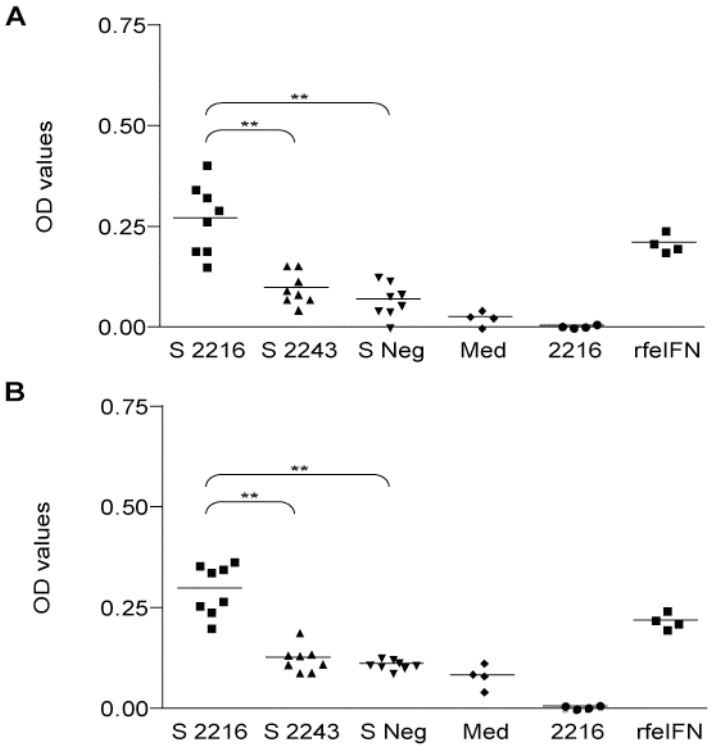


Fig 4.10. Viral inhibition 72 hours post treatment of fcwf-4 cells with supernatants derived from ODN 2216-stimulated PBMCs
fcwf-4 cells were incubated for 72 hours with supernatants derived from PBMCs stimulated with ODN 2216 (S 2216), ODN 2243 (S 2243) or endotoxin-free PBS (S Neg), or with medium only, or with ODN 2216 directly (2216) or with 100U recombinant feline IFNα (rfeIFNα), prior to inoculation with VSV (A) or FCV (B). Supernatants from the PBMCs of 8 adult cats were used. Each dot represents mean optical density (OD) values from spectrophotometric readings of plaque assays conducted on duplicate wells treated with supernatants from an individual cat. ** $p<0.01$

PBMCs stimulated with IRMs *in vitro,* however, liberate soluble molecules that hold the potential to support the enhancement of defence mechanisms in the mentioned feline cell lines. Out of the four IRMS tested in our experiments, only ODN 2216 could induce potent enough production of antiviral molecules by PBMCs. Indeed, when supernatants of ODN 2216-treated PBMCs were incubated with fcwf-4 and CrFK cells, they increased resistance of these cells to FCV, FHV, FCoV and FPV. In both cell lines, the induction of Mx highly correlated with viral inhibition, supporting the hypothesis that the various sybtypes of type I IFN present in the supernatants are most probably responsible for the observed repression in replication of these viruses. The distinct sensitivities to rfeIFNα indicated by both cell lines were reflected in their differential induction of Mx and viral inhibition in response to treatment with the supernatants. Notably however, although fold inhibition of the individual viruses was much lower in CrFK cells, the ranking of the viruses from most to least inhibited was the same in both cell lines. Only the correlations of viral inhibition rates with expression of Mx in the target cells did not quite overlap for both cell lines, an observation that could be due to a disproportional sensitivity of the cells to the individual viruses. In turn, supernatants of ODN 2216 treated PBMCs could

induce antiviral mechanisms and repress FeLV replication in FEA cells, extending the observataions to an additional cell line and an additional virus.

Importantly, the enhanced resistance to infection of at least one virus (FCV) lasted a minimum of 3 days following treatment with the supernatants from ODN 2216-treated supernatants. Even though this experiment was conducted with the feline virus that was most sensitive to the supernatants, these observations further support the feasibility to induce short-term resistance to viruses *in vivo*.

5. Discussion

The initial aims of this study included developing tools to measure hallmarks of innate immunity in the domestic cat and further utilizing these tools to characterize early immune responses to viral infection both *in vitro* and *in vivo*. It was additionally foreseen to assess the possibility to manipulate innate immunity with synthetic molecules called IRMs, so as to increase resistance of the host to viral infections. The feasibility of "teflonization", or unspecific protection against infection by various genetically different viruses simultaneously, was furthermore to be tested in the feline model. The study of innate immune responses confers crucial information regarding the first encounter between a pathogen and host immune cells. We anticipated that experiments with newly developed PCR assays enabling to measure the expression of a series of genes related to the organism's first line of defence would provide valuable information regarding both host and viral factors playing an important role in the early defence mechanisms against individual feline viruses. We expected that IRMs that have already shown immunomodulatory properties in mice could also affect the behaviour of feline immune cells and set out to characterize eventual differences between these species. Furthermore, we hypothesized that potent IRMs could increase natural antiviral defence networks to a threshold that would confer at least partial protection to several viruses simultaneously. The main results obtained in this study are presented in the manuscripts of section 3 and thoroughly discussed in the corresponding discussion sections. In this chapter, the general findings and challenges of this project will be first summarized. With the perspectives of current knowledge in the field of innate immunity to viruses in humans, mice and cats, the overall achievements of this study and relevance to researchers in the field will then be discussed. Finally, prospects for the future experiments will be presented.

5.1 General findings and challenges

In order to facilitate the evaluation of innate immune responses in the domestic cat, PCR assays enabling the measurement of the expression of 12 genes were first developed. Species-specific sequences for the dometic cat were retrieved from Ensembl

(www.ensembl.org) and GeneBank (www.ncbi.nlm.nih.gov) for the genes encoding relevant TLRs, various cytokines, including all subtypes of feline type I IFN, proinflammatory and natural killer cell (NK) related cytokines, and the intracellular antiviral Mx protein. All these genes are known to play important roles in the network of immune cascades responsible for initial responses to viral invasion. Together with additional assays previously developed in our laboratory enabling to discern various T helper (Th) responses, these tools then enabled to cover essential aspects of early immune responses to various feline viruses. Importantly, the amplification efficiency of all systems utilized in this study approximated 1 (Table 2, manuscript 1), a crucial requirement when analyzing the relative expression of a gene under varying cellular circumstances with the comparative Ct method [75].

In the new possession of tools to measure alteration in the gene profiles related to early immune mechanisms in the cat, the responses to viral inoculation *in vitro* of several feline cell lines as well as of PBMCs isolated from the blood of domestic cats could be evaluated. The initial results presented in manuscript 1 clearly indicate the promotion of different immune mechanisms by different viruses. Strength, breadth and kinetics of early immune responses elicited by feline cells could be evaluated, and hints on the potential evolution of strategies to circumvent host antiviral defences by certain viruses were revealed. However, the obtained data obviously represent only a first step in the characterisation of innate defence mechanisms to these viruses, as further experiments are necessary to determine the significance of individual properties of cells and tissues as well as virus-related factors such as source and infectious dose. Also, although the induction of systemic type I IFN responses upon FIV infection *in vivo* could be successfully measured in the blood of a group of experimentally infected kittens, the modulation of other factors of innate immune mechanisms could however not be detected, indicating some challenges in the use of the developed systems for measurements *in vivo*. As innate immune responses occur rapidly and often at specific peripheral sites following viral encounter, both the time point and the localization of the cells or tissues collected for measurement are of primordial importance. Thus, particularly in the case of viral diseases occurring essentially at specific sites such as feline herpes virus infections, systemic innate immune responses may be inexistent or too

weak to detect due to insufficient sensitivity of the developed PCR assays. Measurements should then ideally be carried out in local lymphoid tissues, a necessity that could lead to difficulties when the kinetics of a response need to be determined and samples have to be collected at various time points over a short period of time. Additional experiments will reveal the true potential of the newly designed PCR systems in research linked to feline virology and immunology.

A series of *in vitro* experiments further enabled to assess various IRMs for their immunomodulatory potential in the domestic cat. Stimulators for each TLR efficiently recognizing natural viral invasion were chosen according to popularity for other species in the literature: Poly IC, a synthetic double-stranded polyriboinosinic–polyribocytidylic acid, most popular synthetic analogue to dsRNA and stimulator of TLR3 [76], resiquimod (R-848) an imidazoquinoline that binds to TLR7/8 [77], and synthetic CpG molecules, which trigger TLR9 [78]. As representatives for the latter group, dSLIM™ was selected for its innovative structure with many demonstrated advantages *in vivo* [79, 80] and ODN 2216, the prototype of class A CpG molecules, for its particularly potent induction of type I IFN previously reported in murine studies [81] (for more information concerning different CpG classes, see manuscript 2). In contrast to direct initiation of antiviral mechanisms by a recombinant IFNα protein, it was foreseen that stimulation with an IRM could confer biological advantages through the induction of a wide range of type I IFN subtypes, which have been shown to possess differential antiviral properties [82]. Although the effects of all these IRMs have been tested in the context of disease, their prophylactic potential against viral infections as stand-alone agents, not to mention the concept of “teflonization” (see section 2.1.3), remains under-explored. In this study, the application of all IRMs induced immunomodulation in feline immune cells, however not always with the expected potency. It became rapidly clear that the toxicity of Poly IC for feline cells would highly restrict the utilization of this IRM in the feline host, and that the antiviral responses elicited by both R-848 and dSLIM™ were not strong enough to inhibit viral replication. ODN 2216, on the other hand, enhanced expression of the highly antiviral type I IFN genes of up to 12’000 fold in feline PBMCs within 24 hours (Fig. 3, manuscript 2), indicating powerful antiviral properties. A broad analysis of the immunomodulatory effects of ODN 2216 was consequently initiated, including

assessment of the responses elicited by various cell types, determination of optimal concentrations and analysis of the kinetics of the induced immune responses. Also, the collection of available PCR assays enabled to characterize the gene profile alterations upon treatment of PBMCs from fourteen individual cats belonging to age groups varying between 10 weeks and 14 years of age. Additional experiments then enabled to further characterise the network of interactions induced by ODN 2216 within players of innate immunity: significant proliferation of lymphocytes was observed in a tritium thymidine incorporation assay and upregulation of co-stimulatory molecules on the surface of both lymphocytic and non-lymphocytic immune cell populations was detected by flow cytometry. The expression of genes encoding for NK cell stimulator IL-15 and effectors IFNγ and Granzyme B were increased after the treatment of PBMCs with ODN 2216 (Fig. 3, manuscript 1) seemed to predict enhanced cytotoxic potential of stimulated NK cells. This was accordingly tested with a conventional NK cell cytotoxicity assay, in which human erythroleukemia K562 target cells were pulsed with ^{51}Cr prior to their incubation with ODN 2216-stimulated PBMCs as effectors. Although this assay is normally carried out with purified NK cells, successful results when utilizing PBMCs as effector cells have been reported [83]. Unfortunately, despite several attempts in optimizing the various influential factors within the assay, the isolated feline PBMCs could not be stimulated to kill the target cell line, even when IL-2, the commonly employed NK-stimulatory cytokine, was used.

Another crucial aspect in the natural combat against viruses is the induction, in yet uninfected cells, of intracellular antiviral proteins that interfere with viral replication and thus prevent productive infection of the cell and viral spread in the host. Many such proteins have been described and their roles in the inhibition of different viruses have been reported. Unfortunately, none of these genes have been sequenced in the feline species to date. One study however described the successful design of primers for conventional PCR based on the human Mx1 sequence [67]. This sequence was consequently also used to develop the real time PCR assay utilized in this study, as its degree of homology with the feline nucleotide sequences seemed relatively high. This assumption was confirmed by the high amplification efficiency obtained during experiments aiming at determining optimal primer and probe concentrations to be used for this system (E=1.00, Table 2, manuscript 1). The new availability of this PCR assay

proved to be extremely useful in this study, as the expression of Mx can readily be used as a hallmark for the upregulation and biological activity of type I IFN [60], which could not be detected on a protein level due to the lack of specific antibodies available on the market. It turned out that ODN 2216 not only induced high levels of type I IFN in feline PBMCs, but also positively affected the transcription of Mx in these cells, supporting the biological activity of the produced type I IFN.
Altogether, the main steps of an innate immune response following stimulation with ODN 2216 are covered in the manuscript 2, including effects of this synthetic molecule on the expression of its natural receptor as well as the modulation of both genetic profiles and phenotypic characteristics that directly and indirectly affect the roles of various cell types.

As the ultimate aim of this study was to determine whether prophylactic IRM treatments could inhibit feline viral replication, the next step consisted in linking the innate immune mechanisms induced by ODN 2216 with the resistance of target cells to infection with various viruses. For this, an *in vitro* method was utilized, in which the supernatants of ODN 2216-stimulated PBMCs were incubated with various feline cell lines prior to their inoculation with a particular virus. The supernatants conferred both CrFK and fcwf-4 cells significant resistance to infection with FCV, FHV, FPV and FCoV, and the spread of FeLV in FEA cells could also be considerably inhibited. These results could moreover be repeated with supernatants obtained from the stimulated PBMCs of up to 8 individual cats, supporting their validity. Admittedly, the utilized supernatants contained a mixture of soluble molecules that could all participate in the total antiviral effects observed. Type I IFN however, seemed to play a crucial role in the enhanced resistance to viruses in these experiments, as the viral inhibition was both comparable to that obtained after direct treatment of the cells with rfeIFNα and highly correlated with the induction of Mx transcription in the cells to be inoculated (manuscript 2 and section 4, Fig. 4.8). Additionally, experiments aiming at the evaluation of the amount of refIFNα required for the prophylactic inhibition of different viruses on the individual cell lines *in vitro* readily indicated that supernatants attaining this threshold could inhibit viral replication, whereas those not containing comparable levels of type I IFN partially or completely failed to decrease viral propagation in the cells (section 4, Fig. 4.8 and 4.9). Although PCR

systems are available in our laboratory to measure virus replication [84-89], this method failed to indicate significant inhibition of FCV, FHV and FCoV in initial supernatant experiments. This was most likely linked to high amounts of residual viral nucleic acids present in the culture medium both originating from the stock virus utilized for *in vitro* inoculation and liberated by cells dying as a result of infection. Actually, despite technical efforts to minimize their presence, the detection by real-time PCR of FeLV RNA and DNA residues in the after removal of the virus from treated cultures can still be visualized in the graphs presented in the manuscript 2 (Fig. 6A). The possibility to detect viral inhibition of this virus by real time PCR most probably resides in its incapacity to induce cytopathic effects (CPE) in the FEA cells used as target, or in unknown viral or cellular factors that were affected in the course of the experiments. As the other feline viruses utilized in this study readily induce CPE in CrFK and fcwf-4 cells, both viral replication and the inhibition thereof were assessed by a plaque assay, which remains unaffected by non-infectious viral nucleic acids. Although in each case viral replication was inhibited and not eliminated, it is important to keep in mind that regardless of the infectious dose utilized, *in vitro* inoculation should be much more aggressive and thus not comparable to the small concentration of virus transmitted in the field. Agents conferring partial protection to viruses *in vitro* may therefore turn out to be much more potent *in vivo*, providing they are able to induce systemic reactions.

All in all, the successful prophylactic induction of resistance to representatives of five different feline virus families simultaneously supports the potential of ODN 2216 to confer the host significant advantages in overcoming viral affections in an early phase. This synthetic molecule thus represents an ideal candidate for *in vivo* "teflonization" experiments of the cat. Although molecules of the same CpG-containing IRM subfamily have shown antiviral effects against viruses in mice [90-93], the prophylactic potential of this particular molecule remains to be discovered in mice, cats and other species.

5.2 Relevance of the study

Admittedly, the mechanisms reported in this study linked to innate immunity, immune modulation by IRMs and viral inhibition have all been described before in the murine model as well as in human cells and thus do not constitute a novelty in the field. Also, it was not news to us that certain feline viruses were sensitive to type I IFN, as various

studies have revealed this already in the past [94-101]. Nevertheless, our findings contribute in many ways to providing new perspectives to research groups focusing on innate immunity and open original paths for future projects in this field.

First, this project greatly supports the development of the cat as an alternative model for studies related to innate immunity to viral infections. As elaborated in section 2.3.2 the feline model holds several advantages in studies concerning innate immunology when compared to laboratory mice: not only does the cat happen to be very susceptible to a wide range of viruses that are biologically similar to those affecting humans, the feline model also enables to carry out both *in vitro* and *in vivo* studies in an outbred species under natural conditions. Along these lines, the data obtained in the present study raises fundamental questions concerning age, individuality, species specificity and feasibility of extrapolation in studies focusing on the use of IRMs in medicine. Also, the sequencing of the entire genome of an Abyssinian cat named Cinnamon in 2006 [102] further supports the consideration of cats as good comparative models in human medicine and, together with the prospects of modern genomics technologies, will bring valuable information concerning the pathogenesis of many diseases. Our group has been significantly contributing to feline research for over 25 years. The tools necessary for the monitoring of both the replication of a large array of feline pathogens and the corresponding immune responses have been established and successfully applied in our laboratory. We strongly believe that raising awareness regarding this animal model and strengthening our knowledge in feline immune responses would represent a great advancement in both human and veterinary medical research.

Second, the domestic cat populations worldwide will greatly profit from this advance in feline immunology. The developed real time PCR assays will certainly support the better understanding of host virus interactions in the feline species. For example, a more detailed investigation of the nature of early immune responses to viruses in animals with different outcomes of infection will confer valuable information regarding effective defence mechanisms and prognosis in individual cats. The promising results obtained *in vitro* in this project should also encourage further studies for the development of more efficient prophylactic and therapeutic strategies against fatal feline viral infections.

Third, this study is to our knowledge the first to present the possibility to inhibit replication of viruses from many different families simultaneously. Indeed, 5 feline viruses belonging to the *Coronaviridae*, *Herpesviridae*, *Caliciviridae*, *Parvoviridae*, and *Retroviridae* families, in addition to the vesicular stomatitis virus (VSV) used as control in the *in vitro* experiments, could all be significantly inhibited after incubation of various cell lines with supernatants of feline PBMCs stimulated with ODN 2216. Moreover, in additional experiments carried out with VSV and FCV, these effects were shown to last at least 72 hours (section 4, Fig. 4.10). In other words, a single treatment of cells with this synthetic molecule as stand-alone agent suffices to induce resistance to viruses with completely different genetic properties for several days *in vitro*. These accomplishments support the feasibility of this experimental setup as model for emerging viral diseases, and uncover a promising future for the "teflonization" of the cat *in vivo* (see section 2.1.3 for definition of "telflonization").

Finally, our data greatly contributes to the validation of an *in vitro* screening method for IRMs that had only been seldom used to date. Many physiological and immunological parameters that greatly influence the success of an immunomodulatory agent cannot be taken into consideration when tested *in vitro*, and answers to such questions are oftentimes searched for directly with *in vivo* experiments. The 3-step system comprising 1) the stimulation of purified PBMCs with an IRM, followed by 2) the transfer of cell-free supernatants onto various cell lines and 3) the inoculation of these target cells with feline virus and measurement of viral replication mimics to a great extent an *in vivo* situation (explained in the discussion of the manuscript 2), and thus supports growing ethical expectations in research with animals [103]. Providing the necessary target cell types are available, this method could be used in preliminary tests when predicting the use of an IRM as vaccine adjuvant, or in preventive or therapeutic settings for various diseases. Both *in vitro* experiments with cells from other species and *in vivo* experiments in the cat will further validate this strategy.

5.3 Limitations and frustrations

The major limiting factor in the present study was the restricted availability of commercial tools for the feline species. Particularly antibodies recognizing feline

proteins could have supported the study of biological properties of viral particles and various mechanisms involved in immune responses of the host. In this way, specific antibodies against feline type I IFN could have facilitated the determination of both the amount of these cytokines present in the supernatants and their role in viral inhibition, while the availability of labelled antibodies of various subtypes recognising different cell surface molecules would have enabled co-staining for different factors on distinct immune cell populations by flow cytometry. Similarly, the investigation of intracellular mechanisms following stimulation with an IRM equally requires the availability of antibodies specific to the proteins involved in the pathways. Another gap to be filled in feline research is the lack of nucleotide sequences for many parameters of immunological reactions. The tedious development of 12 real time PCR assays in this study, enabling to measure the modulation in relative expression of a selection of genes, could hardly confer the information that would be achieved with a microarray. For similar reasons, the overexpression or knockdown of specific proteins cannot be undertaken, because the necessary constructs are not available. Although such restrictions may pressure the development of more innovative concepts in order to demonstrate certain biological aspects, the generation of valuable data is much less efficient, which constitutes a great handicap in a world where the half-life of a scientific idea is shrinking considerably.

5.4 Future perspectives

Considerations for future projects are many. An interesting objective would consist in defining the individual roles of the numerous feline type I IFN subtypes and the different intracellular proteins, particularly Mx, involved in the defence against individual viruses. Furthermore, a series of *in vivo* experiments would enable to determine the clinical relevance of ODN 2216 in the “teflonization” of the cat. Dosage of the molecule as well as route and time point(s) of administration enabling the successful induction and optimal kinetics of a systemic immune response would have to be determined. The induction of immunity at peripheral sites may additionally be required, especially in the context of infections that remain local, such as those induced by the feline herpes virus. Potential safety issues obviously need to be studied. Also, as the structure of ODN 2216 may render the molecule sensitive to nucleases *in vivo*, stabilizing strategies such as biodegradable microparticles [104] and lipid-based delivery systems [105] may also need

to be taken into consideration. Protection against infection by several viruses should also be demonstrated experimentally, ideally in the context of viral transmission by shedding to naïve cats. Finally, similar experiments conducted with cells and viruses specific to other species could give further indications regarding the feline model and possibilities to extrapolate findings to other animal populations and to humans.

5.5 Conclusions

In an era of permanent emergence of new health-threatening viral diseases such as the severe acute respiratory syndrome (SARS) and avian influenza, research groups worldwide are concentrating on elaborating the recent idea of inducing innate immune responses as prophylactic or therapeutic measures in the setting of infectious diseases. As many human and animal diseases share a pathogenic basis, and although veterinary species need advances in biomedical research for their own interests, the availability of an appropriate comparative small animal model for such research purposes is therefore currently highly desirable. It is today widely accepted that the future of many areas of human and veterinary biomedical research is very much interdependent.

The present work is thus in line with the scientific trend of this decade. The domestic cat represents a powerful model in the analysis of immune responses to viruses, and the development of specific tools enabling to characterize innate immune mechanisms in this species readily supports further use of this outbred model for studies regarding innate host-virus interactions. Our study further suggests that non-specific prophylactic manipulation of the cat's early immune mechanisms holds the potential to induce resistance to infection by viruses with a wide range of genotypic and phenotypic properties. As evolutionary considerations render the elaboration of preventive measures to feline viral infections particularly challenging, these results create an exciting new opening for the development of novel short-term antiviral preventive measures in both human and veterinary medicine.

6. References

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